# STUDIES ON INHIBITOR RESISTANCES IN THE YEAST *SACCHAROMYCES CEREVISIAE*

by

E.J. Griffiths 1975

A Thesis submitted to the University of Warwick for the Degree of Doctor of Philosophy.

This research was carried out mainly in the Department of Molecular Sciences, University of Warwick. Some of the results were obtained at the New York Institute of Public Health Inc., New York City.

CONTENTS

## FIGURES

TABLES

TABLES

ACKNOWLEDGEMENTS

I would like to acknowledge the advice and encouragement given to me by my supervisor Dr. D.E. Griffiths, during the course of this work. I would also like to thank Dr. A. Tzagoloff for his hospitality during June and July 1972 and for much subsequent useful advice. Many thanks are owed to my colleagues in the Department of Molecular Sciences for much useful discussion, advice and encouragement. The receipt of a Medical Research Council postgraduate training fellowship is acknowledged.

## ABBREVIATIONS

| | |
|---|---|
| $ad_2$ | adenine |
| arg | arginine |
| ADP | adenosine diphosphate |
| ATP | adenosine triphosphate |
| ATPase | adenosine triphosphate phosphohydrolase (3.6.1.4) |
| BPB | bromophenol blue |
| CAP | chloramphenicol |
| CHI | cycloheximide |
| C : M | chloroform : methanol 2 : 1 v/v |
| DCCD | dicyclohexylcarbodiimide |
| Dio-9 | an inhibitor of cold sensitive $F_1$ ATPase |
| DNA | deoxyribonucleic acid |
| EDTA | ethylene diaminetetraacetic acid |
| Er | erythromycin |
| E.S.R. | electron spin resonance |
| $F_1$ ATPase | cold labile, oligomycin insensitive, aurovertin, Dio-9 sensitive enzyme first isolated by Pullman *et al.* (1960) |
| $F_9$ | proteolipid subunit 9 of the O.S. ATPase |
| His | Histidine |
| mDNA | mitochondrial deoxyribonucleic acid |
| met | methionine |
| NEM | N ethylmaleimide |
| $NH_4Br$ particles | SMP depleted of $F_1$ ATPase and OSCP |
| O.S. ATPase | oligomycin sensitive, cold stable ATPase |
| OSCP | oligomycin sensitivity conferring protein |
| OL | Oligomycin |

| | |
|---|---|
| Pi | inorganic phosphate |
| PRS | post ribosomal supernatant |
| $\rho$ | 'rho' factor taken to be the mitochondrial genome |
| SDS | sodium dodecyl sulphate |
| SMP | sub mitochondrial particles |
| S. cerevisiae | Saccharomyces cerevisiae |
| TCA | trichloracetic acid |
| TEA | buffer containing 10 mM Tris-acetate, pH 8.0, 2 mM ATP, 1 mM EDTA |
| TET | triethyl tin |
| TLC | thin layer chromatography |
| Trp | tryptophan |

## SUMMARY

A series of mutants were selected on their ability to grow in the presence of the inhibitor of oxidative phosphorylation, oligomycin. The work described in this thesis was an attempt to identify the aberrant peptide(s) responsible for an increase in the resistance to oligomycin in the case of A21 and A15 and also to venturicidin and TET in the case of A19. The factor(s) responsible for these phenomena were shown to be located in an ATPase and OSCP depleted membrane fraction. The mitochondrial membranes from both wild type and mutants were shown to be structurally similar by E.S.R. and $^{3}$H-NEM labelling. A soluble inhibitor sensitive ATPase (O.S. ATPase) was prepared from both wild type and mutant strains. The enzyme from the mutants studied showed no aberrations during purification or large scale deletions upon analysis by polyacrylamide gel electrophoresis. The enzyme from both wild type and mutant cells was shown to possess a site of action of oligomycin, venturicidin and TET. The solubilised enzymes were shown to have retained the specific resistance phenomena found at the level of SMP in the mutants studied. One of the mutants, A21, was shown to have aberrant kinetics of formation of inhibitor sensitive ATPase after incubation in CAP. Analysis of the mitoribosomal products over a time course after incubation in CAP was made on wild type and A21 cells. Antisera to O.S. ATPase was used to identify the mitoribosomal products associated with O.S. ATPase. The major mitoribosomal product, subunit 9 of the O.S. ATPase, was purified and partially characterised. Problems concerning the solubility of this product precluded further analysis of it. The appendix describes work carried

out on a preparative gel electrophoresis apparatus.

# CHAPTER 1

# INTRODUCTION

## 1.1 YEAST - A SUITABLE SUBJECT FOR THE STUDY OF BIOCHEMICAL GENETICS OF OXIDATIVE PHOSPHORYLATION

The classical biochemical approaches to the problem of pathway elucidation, such as purification of pathway intermediates and enzymes and reconstitution of the pathway sequence from these purified constituents, have, for many years, been resisted by the sequence of chemical reactions responsible for energy transformation in mitochondrial membranes.

The major difficulty in using the classical approach to this problem is that the catalytic units carrying out the chemical reactions form an integrated part of the highly organised, lipoprotein system known in eukaryotes as the inner mitochondrial membrane. Difficulties arise in defining the limits of the catalytic units and any perturbation of one unit is bound to have a myriad of repercussions on other units.

Auxotrophic mutants have been extensively used as a tool for the elucidation of biochemical pathways and to apply the mutant approach to the problem of energy conservation would seem to be a logical progression. Application of the mutant approach to biochemical problems has usually employed the use of bacteria, but only recently have bacterial mutants begun to be used in studies of the biochemical genetics of oxidative phosphorylation and this area of study is now undergoing rapid expansion (Gibson, 1971; Cox and Gibson, 1974).

The organism first employed in such studies was a eukaryote, yeast. Yeast is a simple organism, it possesses

mitochondria with properties very similar to those of mammalian mitochondria. It has long been one of the most useful micro-organisms to man and partially as a result of this its genetics, biochemistry and cytology have been extensively studied. The first mitochondrial mutants were identified because of the inability of some yeast cells to grow on non-fermentable substrates but produce a colony of reduced size on limiting fermentable substrate, i.e. petite colonies. These mutants were later found to have survived large lesions or indeed complete deletion of the mitochondrial genome. Many yeasts can survive such genetic damage because a tandem energy supply generated by the glycolytic pathway is open to them. Thus one can dramatically alter the genotypic and phenotypic properties of ~~the~~ mitochondria of yeast and still maintain a viable cell. This coupled with the advantages listed below led to the choice of S. cerevisiae as the organism best suited to the biochemical genetic approach to the problems of energy transformation. The advantages of S. cerevisiae are:-

1) The species is easily cultured, grows as a reasonable rate as a haploid cell in a chemically defined medium and is non-pathogenic.

2) The biochemistry of the species is well known.

3) It is commercially available in large quantities as brewers or bakers yeast, this can be a great convenience when comparatively large amounts of material are required for such techniques as production of antisera.

4) The organism is amenable to cytological observation and manipulation.

5) The genetics of the species has been extensively studied and

methos for its genetic manipulation are available.

6) Methods of isolation of mitochondria from S. cerevisiae are well documented and widely available.

Minor disadvantages have come to light with the particular strain, D22, employed in most of these studies in that the cell flocculates early in the growth cycle under conditions of glucose repression and the cells are difficult to break mechanically or enzymically especially in the stationary phase of growth. This may turn out to be a major disadvantage in studies on the adenine nucleotide translocase system in this strain.

## 1.2 THE PETITE MUTATION

These are the most easily available mutants of S. cerevisiae and have been extensively studied by Ephrussi and co-workers. These mutants occur spontaneously, the cytoplasmic petite occurring with a very high frequency $1 \times 10^{-1}$ to $1 \times 10^{-3}$ compared with a nuclear petite mutational rate of around $1 \times 10^{-6}$ to $1 \times 10^{-7}$ (Ephrussi et al. (1955)). These mutation(s) give rise to colonies distinctly smaller than those of the wild type if both cells were grown aerobically on a mixture of high non-fermentable and limiting fermentable substrate concentrations and the mutations were stable over any number of cell generations (Ephrussi et al. (1955)). The petite colonies grew at a slower rate aerobically on glucose than did the wild type but at a similar rate when grown anaerobically suggesting that the petites had normal fermentative capability but an impaired respiratory system.

It is now clear that cytoplasmic petites do not form a

single class of mutants and that different lesions or complete deletion of the mitochondrial DNA gives rise to superficially similar phenotypes in being respiratory deficient. Petites are now known to differ in the amount and base composition of their mDNA, the degree of retention of genetic material as measured by the deletion or retention of antibiotic resistant mitochondrial genes and their degree of suppressiveness (Ephrussi et al., 1955; 1966).

Mitochondria from a haploid petite mutant have been investigated by Perlman and Mahler (1970a, b; 1971). Their content of ATPase was comparable with that found in the wild type but was unusual in that it was cold stable but oligomycin insensitive and Dio-9 sensitive. These mitochondria showed the multiple lesions in the respiratory chain usually associated with the petite mutation.

Another unusual feature of the petite mutation is its pleiotropic effect and an important question yet to be answered is how does a mutation anywhere along the mDNA forthe most part give rise to a petite phenotype showing a complete lack of mitochondrial protein synthesis. Either a lesion large enough to give rise to the petite phenotype renders survival of a functional mitochondrial protein synthesising system impossible or the regulatory system of the mitochondrial protein synthesising system is so closely intra-related that disruption in one part of it renders the remainder inoperable; the fact that the Hela cell mitochondrial genome is transcribed as a single cistron (Aloni and Attardi, 1971) lends some support to this hypothesis.

## 1.3 CYTOPLASMIC MUTANTS RESISTANT TO ANTIBIOTICS

The petite mutation was the first cytoplasmic mutant to be extensively studied but was strictly limited because respiratory deficient diploids could not be induced to sporulate and so were not amenable to tetrad analysis. However more recently it has been shown that respiratory deficient diploids can sporulate immediately after induction. The discovery of a new class of cytoplasmically inherited mutations in yeast, those conferring resistance or decreased sensitivity to antibiotics has greatly advanced the study of mitochondrial genetics and is now and will in the future provide a very promising approach to the identification of mitochondrial gene products and to the elucidation of the mechanisms of oxidative phosphorylation.

(Kuenzi et.al J. Bact.(1974) 117 80)

When S. cerevisiae is grown on a non-fermentable substrate such as glycerol the organism is obliged to use the mitochondrial respiratory system for the production of energy for growth, thus any inhibitor affecting the respiratory system or affecting the functional integrity of the mitochondria will hamper growth of the organism on such substrates. Thus under these conditions it has proved possible to detect mutant strains of yeast which display a lowered sensitivity or resistance to such inhibitors (Wilkie et al., 1967; Linnane et al., 1968; Wilkie, 1970). Such a resistance may be due to a number of possible changes such as a change in membrane permeability, a detoxification mechanism or an altered binding site for the inhibitor. These mutants can be shown to exhibit cytoplasmic inheritance in that they show mitotic segregation in crosses of the type $I^R\rho^+ \times I^S\rho^+$ and that on sporulation of the diploids the asci contain either

0 : 4 or 4 : 0 resistant : sensitive ascospores. Experiments collating loss of resistance with conversion of $I^{R}\rho^{+}$ strain to $\rho^{-}$ strain have allowed localisation of the cytoplasmic determinants on the mDNA (Linnane et al., 1968; Coen et al., 1970).

Much of the initial work on antibiotic resistance phenomena utilised antibiotic inhibitors of mitochondrial protein synthesis such as chloramphenicol, erythromycin and tetracyclines. None of the mutations impaired mitoribosomal function, the growth rates and the mitoribosomal activity of the mutants being comparable with those of the wild type.

Cytoplasmic Mutants Resistant to Inhibitors of the Reactions of Energy Transformation

Mitochondria and sub mitochondrial particles of S. cerevisiae contain a $Mg^{2+}$ dependent ATPase usually assumed to be the enzyme employed in the final stages of energy conservation in which role it functions as a ATP synthetase. Oxidative phosphorylation and subsequently hence ATPase activity is inhibited in coupled mitochondria by a number of inhibitors, most important to this study are oligomycin (Tzagoloff et al., 1968), venturicidin (Langcake et al., 1974) and triethyl tin (Aldridge and Cremer, 1955). The isolation of mutants resistant to these inhibitors may, therefore, provide a genetic probe into the nature and site of action of these inhibitors on oxidative phosphorylation and may provide information on the assembly and organisation of the mitochondrial energy conservation system.

Several studies have been made of oligomycin resistant mutants encompassing both nuclear and cytoplasmic mutations.

Wakabayashi and Gunge (1970) isolated several cytoplasmically inherited oligomycin resistant mutants one of which was not linked with the rho factor and displayed an unaltered mitochondrial ATPase, other mutants displayed an altered ATPase but the increased resistance of the enzyme to oligomycin was not very marked (Wakabayashi, 1971). Oligomycin resistant mutants of S. cerevisiae have also been isolated by Shannon et al. (1973). The mitochondrial ATPase of the cytoplasmically inherited mutants displayed altered sensitivity to oligomycin though only a two fold increase in the amount of oligomycin required to inhibit the ATPase activity 50% is shown by these mutants.

A series of mutants of S. cerevisiae resistant to high levels of oligomycin were isolated in this laboratory. These mutants were initially divided into two classes; class 1 mutants displayed cross resistance to all inhibitors tested and class 11 mutants which displayed specific resistance to oligomycin and closely related inhibitors such as ossamycin and peliomycin (Avner and Griffiths, 1970; 1973a).

Studies on the class 11 mutants revealed that they showed typical cytoplasmic inheritance (Avner and Griffiths, 1973). The resistance phenomenon was conferred by two groups of distinct non-allelic loci situated on the mitochondrial genome and designated OL1 and OL11, strains D22A21 and D22A15 respectively in this study. The ATPase activity of both mutants is markedly more resistant to oligomycin inhibition than the wild type, OL1 more so than OL11 (Griffiths and Houghton, 1974). Evidence will be presented in this study to demonstrate that these mutations manifest themselves as an

altered protein inhibitor binding site rather than a change in non specific binding or a permeability or regulatory effect. These two loci show a high degree of recombination with each other and in a series of crosses involving mutants at OL1 or OL11 and a series of $\rho^-$ petites variously deleted in known mitochondrial genes has shown that the two oligomycin resistance loci are seperable and the transcribed and translated mutation leads to two altered peptides. The expression of these mutations will be shown to be present in the purified ATPase thus two of the four mitochondrially synthesised subunits of the oligomycin sensitive ATPase (Tzagoloff and Meagher, 1972) may be altered in these mutants.

More recently a third locus conferring oligomycin resistance has been mapped and designated OL111 (Griffiths et al., 1975). This mutant, which is also cross resistant to venturicidin and triethyl tin and is represented by D22A19 in this study, maps very closely to OL1 and it is probable that OL1 and OL111 are located on the same cistron and may express themselves in the same peptide. The specificity conferred by different mutations in the same peptides provides strong evidence that the resistance phenomena are functional and reside in the ATPase complex.

Biochemical studies on triethyl tin resistant, cytoplasmically inherited mutants (Lancashire and Griffiths, 1975) have not progressed as rapidly as those with oligomycin resistant mutants. All attempts to detect an altered ATPase in a mutant selected as resistant to triethyl tin have failed. However evidence will be presented to show that the inhibitory site of triethyl tin inhibition and resistance is on the purified oligomycin sensitive ATPase. Indirect evidence indic-

ating the location of triethyl tin resistant phenomenon on the ATPase will be given utilizing sets of isonuclear diploids isolated from two genetic crosses resulting in genotypes $O^{R}T^{R}$, $O^{S}T^{S}$, $O^{R}T^{S}$ and $O^{S}T^{R}$. Evidence is also furnished from this study for the close spatial interaction between triethyl tin and oligomycin binding sites.

## 1.4 INHIBITORS OF OXIDATIVE PHOSPHORYLATION

Inhibitors selected for use as a genetic probe in studies on the structure and mechanism of the oxidative phosphorylation complex located on the inner mitochondrial membrane should be capable of satisfying the following criteria:-

1) The inhibitor should have a well defined locus of action on the oxidative phosphorylation complex.

2) The site of action of the inhibitor should be predominantly (preferably exclusively) on the mitochondrial inner membrane.

3) The inhibitor should be metabolically inert and not prone to detoxification mechanisms.

The proposed points of action of the inhibitors used in this study, on oxidative phosphorylation are outlined in Fig. 1.1.

### Oligomycin

The antibiotic oligomycin has been widely used as an inhibitor of oxidative phosphorylation in studies on mitochondria (Huijing and Slater, 1961), sub mitochondrial particles (Lee and Ernster, 1968) and purified enzymes (Tzagoloff et al., 1968a). As the first specific inhibitor acting at the site of coupling between respiration and phosphorylation oligomycin has become widely used as a tool in studies on these processes.

ating the location of triethyl tin resistant phenomenon on the ATPase will be given utilizing sets of isonuclear diploids isolated from two genetic crosses resulting in genotypes $O^{R}T^{R}$, $O^{S}T^{S}$, $O^{R}T^{S}$ and $O^{S}T^{R}$. Evidence is also furnished from this study for the close spatial interaction between triethyl tin and oligomycin binding sites.

## 1.4 INHIBITORS OF OXIDATIVE PHOSPHORYLATION

Inhibitors selected for use as a genetic probe in studies on the structure and mechanism of the oxidative phosphorylation complex located on the inner mitochondrial membrane should be capable of satisfying the following criteria:-

1) The inhibitor should have a well defined locus of action on the oxidative phosphorylation complex.

2) The site of action of the inhibitor should be predominantly (preferably exclusively) on the mitochondrial inner membrane.

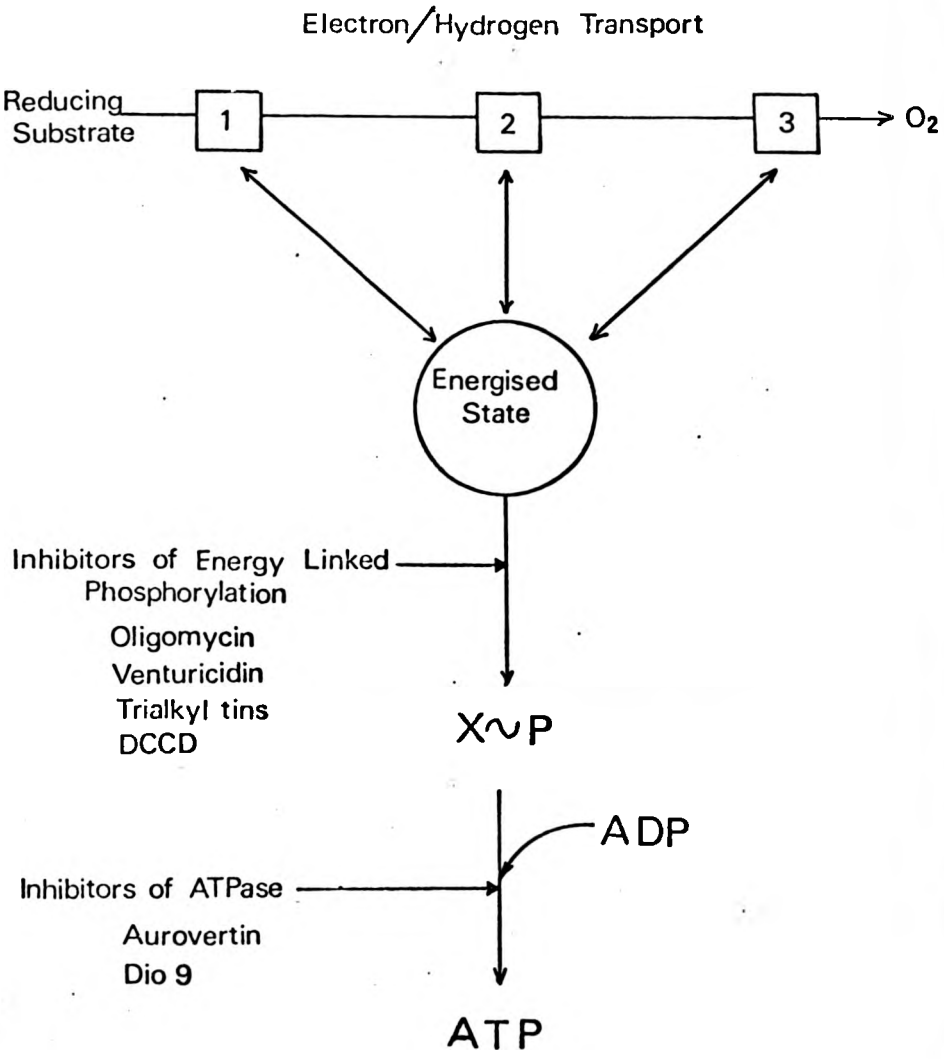
3) The inhibitor should be metabolically inert and not prone to detoxification mechanisms.

The proposed points of action of the inhibitors used in this study, on oxidative phosphorylation are outlined in Fig. 1.1.

### Oligomycin

The antibiotic oligomycin has been widely used as an inhibitor of oxidative phosphorylation in studies on mitochondria (Huijing and Slater, 1961), sub mitochondrial particles (Lee and Ernster, 1968) and purified enzymes (Tzagoloff *et al.*, 1968a). As the first specific inhibitor acting at the site of coupling between respiration and phosphorylation oligomycin has become widely used as a tool in studies on these processes.

Figure 1.1




Schematic Representation of the Sites of Action of some Inhibitors of Oxidative Phosphorylation.

The mode and precise site of action of oligomycin is not yet known. Reviews of the properties of the oligomycins have been presented by Shaw (1967) and Slater and Ter Wells (1969) and a three dimensional structure of oligomycin B determined from single crystal X-ray diffraction data has been proposed by Glehn et al. (1972).

Oligomycin and the related antibiotic rutamycin are isolated from species of Streptomyces, oligomycin from Streptomyces diastochromogenes (Smith et al., 1954) and rutamycin from Streptomyces rutgersensis (Thompson et al., 1961). Both compounds are very similar in their chemical, physical and biological properties but can be separated from each other by chromatography (Shaw, 1967).

Preparations of oligomycin contain three related alcohols, A, B and C which vary in their molecular weight from 397 to 480 (Masamure et al., 1958). All the oligomycins inhibit oxidative phosphorylation (Lardy et al., 1964) the order of effectiveness being A>B>C (Lardy et al., 1965). The effect of oligomycin on mitochondria is to inhibit coupled respiration and mitochondrial ATPase activity, induced or otherwise, provided that the $F_1$ ATPase remains membrane bound. It also inhibits $^{32}$Pi-ATP exchange reactions and the exchange of $^{18}O$ between Pi and $H_2O$ in rat liver mitochondria. Oligomycin also inhibits energy linked reversal reactions when ATP is the energy source but not when energy is furnished by high energy intermediates either on the main path of oxidative phosphorylation (Danielson and Ernster, 1963) or derivable from such intermediates (Lardy et al., 1964) i.e. when energy is conserved by respiratory chain substrate oxidation.

The effect of oligomycin on beef heart submitochondrial particles has been shown to be of a more complex nature(Lee and Ernster, 1968). At low oligomycin concentrations, 0.2 to 0.4 µg/mg protein, oligomycin stimulates oxidative phosphorylation, its reversal and $^{32}$Pi-ATP exchange reactions in particles prepared by sonication in the presence of EDTA but not in particles prepared in the presence of ATP and $Mg^{2+}$. In this case oligomycin has an inhibitory effect on oxidative phosphorylation and $^{32}$Pi-ATP exchange. Lee and Ernster (1968) concluded that oligomycin acts on the respiratory chain linked energy transfer system of EDTA particles in two ways, at low concentrations it inhibits the hydrolytic cleavage of a non phosphorylated high energy intermediate which is common for the three energy coupling sites of the respiratory chain and at higher concentrations it inhibits the interaction of the same intermediate with Pi and ADP to form ATP. Oligomycin has no effect on bacterial systems and only at high concentrations on photophosphorylation where it acts as a weak uncoupling agent (Avron and Shavit, 1965).

Triethyl Tin (TET)

Triethyl tin is a member of a range of compounds known as the organo tins, many of which are toxic to living organisms and their toxicity varies considerably with the number and type of organic groups in the compounds; trialkyl tin derivatives being the most toxic and trioctyl derivatives being essentially non toxic. Attempts to discern the biochemical mode of action of the alkyl tins responsible for their toxicity led to the conclusion that the trialkyl derivatives had a different

mode of action from other derivatives. Whereas diethyl tin was primarily an inhibitor of $\alpha$-oxo acid oxidases (Aldridge and Cremer, 1955) the trialkyl tin compounds appeared to be effective inhibitors of coupled respiration and phosphate esterification in respiring mitochondria (Aldridge and Cremer 1955; Aldridge, 1958; Aldridge and Threlfall, 1961; Sone and Hagihara, 1964). This inhibitory action of trialkyl tins appeared to be very similar to the action of oligomycin as they also inhibited partial reactions such as $^{32}$Pi-ATP exchange and ATPase activity (Aldridge and Street, 1964). These properties commend triethyl tin as a candidate inhibitor for the production of organisms mutated in their oxidative phosphorylation mechanism (Lancashire and Griffiths, 1975).

Triethyl tin binding to rat liver mitochondria suggested two binding sites, one of high affinity, 1 x $10^5$ to $10^6$ $M^{-1}$, and a second site of lower affinity (Aldridge and Street, 1970). It was thought that oligomycin and triethyl tin acted with different modes of action through different sites as it was observe that triethyl tin could also inhibit uncoupler stimulated respiration. An important advance in this study was the demonstration that trialkyl tin compounds could mediate transport across mitochondrial membranes of halide and thiocyanate ions in exchange for hydroxyl ions (Selwyn et al., 1970). In a sucrose medium free from anions which are effective in the anion-hydroxide exchange catalysed by trialkyl tins, that is anions essentially present in the usual .1M KCl suspension buffers, (Aldridge, 1957; Aldridge and Street, 1971) the effects of trialkyl tins on mitochondria are similar to those of oligomycin in that they inhibit coupled oxidative phosphorylation but do

not inhibit uncoupler-stimulated respiration.

In a medium containing KCl similar oligomycin-like effects are observed but in addition some release of respiratory control and marked swelling of the mitochondria occur. A time dependent inhibition of uncoupler stimulated respiration seems to be a secondary effect of mitochondrial swelling. The concentration of trialkyl tin required to produce uncoupling correlates with the concentration required to mediate the anion-hydroxide exchange. Uncoupling by trialkyl tins is attributed to the action of the anion-hydroxide exchange followed by leakage of the anion out of the mitochondria resulting in discharge of both pH differential and electrical potential difference across the mitochondrial membrane.

Dicyclohexylcarbodiimide (DCCD)

DCCD has been shown to be a potent inhibitor of oxidative phosphorylation and of ATP driven partial reactions of oxidative phosphorylation and thus appears to act in a manner analogous to that of oligomycin. These inhibitory effects are immediate at high concentrations of DCCD (40 nanomoles DCCD/mg protein) (Beechey et al., 1966), however prolonged incubation of mitochondrial preparations with DCCD produces inhibitory effects at much lower concentrations (1 nanomole/mg protein). In contrast to oligomycin these time dependent inhibitory effects are not reversible and DCCD, after incubation, appears to covalently bind the mitochondrial preparation at, or very close to the site of inhibitory action(Beechey et al., 1967; Cattell et al., 1970). Catell et al. (1970) and Knight et al. (1968) using $^{14}C$ DCCD have shown covalent inhibitor binding to

mitochondrial proteolipid fractions.

Displaying these properties DCCD is obviously a prime candidate inhibitor for biochemical genetic studies on yeast carrying mutations in the mitochondrial energy conservation system as this would provide a very powerful tool for the possible identification of the mutated binding site. However to date two problems have impeded such investigations; i) the work so far cited on covalent DCCD binding was carried out on ox heart mitochondria and studies so far have shown that while DCCD displays its inhibitory effects on yeast mitochondrial preparations it does not appear to bind covalently to sub mitochondrial particles, purified oligomycin sensitive ATPase or the purified proteolipid fraction associated with the oligomycin sensitive ATPase. ii) Attempts to isolate yeast mutants resistant to DCCD have been impeded by problems associated with complexing of DCCD in the growth media rendering it biologically inactive.

DCCD differs from oligomycin in that it inhibits bacterial oxidative phosphorylation and ATPase activity if the ATPase remains integrated with the membrane. Oligomycin is without effect on these systems.

Venturicidin

Venturicidin is a toxic antibiotic and potent fungicide first isolated by Rhodes et al. (1961) in a search for antibiotics active against apple scab (Venturia inaequalis). It is produced by three distinct strains of Streptomyces and a small scale isolation procedure from Streptomyces griseolus has been described by Langcake et al. (1974). The studies of Walter et

al. (1967) indicated that venturicidin acts in a manner similar but not identical to rutamycin on mitochondrial oxidative phosphorylation. These workers found that the effect of partially inhibitory amounts of rutamycin and venturicidin were at least additive and concluded that the two antibiotics act at the same site. Further studies by Griffiths et al. (1975) on mitochondrial venturicidin resistant mutants have resulted in more complex concepts of venturicidin, oligomycin and triethyl tin binding and their possible interactions at the level of the ATP synthetase complex.

The structure of venturicidin has been established (Brufani et al., 1968; 1972) and has been shown to be a macrolide antibiotic which is different in structure from oligomycin and contains an additional substituted D-rhamnose residue. The structural similarities and similar mode of action of venturicidin and oligomycin suggest a common binding site, a contention supported by genetic data (Griffiths et al., 1975). Griffiths and co-workers suggest that some structural feature of the macrolide ring common to both antibiotics may bind at a common site represented by locus OL111 in strain D22A19. Evidence from studies on triethyl tin resistant mutants indicates a second binding site for venturicidin represented by locus $T_1$ and perhaps binding to the D-rhamnose residue of venturicidin. This possibility was tested by comparing the effects of oligomycin and the aglycone of venturicidin, a derivative of venturicidin which has lost the D-rhamnose residue (Brufani et al., 1968), and should therefore behave like an analogue of oligomycin rather than like venturicidin in its interaction with $OLY^R$ and $VEN^R$ $OLY^R$ mutants, This in fact was found to

be the case (Partis, M.D., private communication). Venturicidin in acting upon S. cerevisiae, appears to have two binding sites, one of which binds to some macrolide ring structure and a second which binds to the D-rhamnose residue.

## 1.5 MITOCHONDRIAL ADENOSINE TRIPHOSPHATE PHOSPHOHYDROLASES (ATPases)

Despite the fact that oligomycin sensitive ATPase is regarded as the functional unit in the terminal enzymatic reaction of oxidative phosphorylation only comparatively recently have efforts been made to study the properties of the purified form of the enzyme rather than reconstituted inner membrane like vesicles of very poorly defined composition which catalyse reactions such as oligomycin sensitive ATPase activity (Racker, 1969) and Pi-ATP exchange activity (Mitchell, 1966). In contrast purified oligomycin sensitive ATPase of defined lipid and protein composition which perhaps can be inserted into vesicles of defined composition should provide a fruitful model with which to test predictions resulting from the various hypotheses forwarded to explain the mechanism of oxidative phosphorylation. Mitochondrial ATPase in its intact form integrated into the mitochondrial inner membrane is an elaborately complex molecule and Fig. 1.1 gives an outline of what is believed to be its basic structure and subunit composition in S. cerevisiae. The enzyme is separable into three functional components, the $F_1$-ATPase which catalyses ATPase activity (Pullman et al., 1960) and in its isolated form is sensitive to aurovertin, Dio-9 and ephrapeptin but not to oligomycin, venturicidin, triethyl tin and DCCD; the oligomycin sensitivity

conferring peptide which forms a single class of peptide and appears to have the function of binding $F_1$ ATPase to the mitochondrial membrane and finally the membrane factor which is the least understood functional component of the enzyme and is thought to direct the flow of protons to $F_1$ ATPase during oxidative phosphorylation (Mitchell, 1973; 1974). A fourth functional group may be represented by the ATPase inhibitor protein though there is still a great deal of controversy over the identity and location of this peptide (Knowles and Penefsky, 1972; Brooks and Senior, 1971).

Oligomycin Insensitive ATPase Preparations

The ATPase enzyme first prepared from bovine heart mitochondria by Pullman et al. (1960) and later from yeast mitochondria (Schatz, 1967; Tzagoloff, 1969a) were oligomycin insensitive, lipid free and water soluble preparations which formed the major component of the oligomycin sensitive complex (Tzagoloff, 1971) and has the unusual property of being cold labile (Penefsky et al, 1965). Analogous preparations have been made from rat liver mitochondria (Caterall and Pedersen, 1971; Lambeth and Lardy, 1971), chloroplasts (Farron, 1970; Howell and Moudrianaris, 1967) and from several bacterial strains (Bragg and Hou, 1972; Schnebli and Abrams, 1970; Munoz et al., 1969). These preparations are completely resistant to most inhibitors which display an inhibitory effect on the membrane bound enzyme with the notable exceptions of aurovertin (Lardy, 1964), ephrapeptin (T.G. Cartledge, Personal Communication) and to a lesser extent Dio-9.

Purification schemes of most $F_1$ ATPase preparations are similar in several respects, they start with membrane fragments

and employ sonication to strip $F_1$ from the membrane and use anion exchange and gel filtration chromatography as terminal purification steps. Most of the schemes used employ ATP containing buffers throughout the purification (Knowles and Penefsky, 1972; Horstman and Racker, 1970; Senior and Brooks, 1970). Since the $F_1$ enzyme is cold labile when removed from the membrane the post-sonication steps of the purification are carried out at room temperature. All these preparations are judged homogeneous by several criteria and exhibit ATPase activities of at least 60 μmoles Pi/min/mg.

The release of analogous $F_1$ ATPases from bacterial and chloroplast membranes can be effected without recourse to sonication (Farron, 1970; Lein and Racker, 1971; Schnebli and Abrams, 1970) and is usually achieved by repeated washings either with low ionic strength buffers or buffers containing EDTA. These preparations are also water soluble and cold labile.

Oligomycin sensitive ATPase Preparations

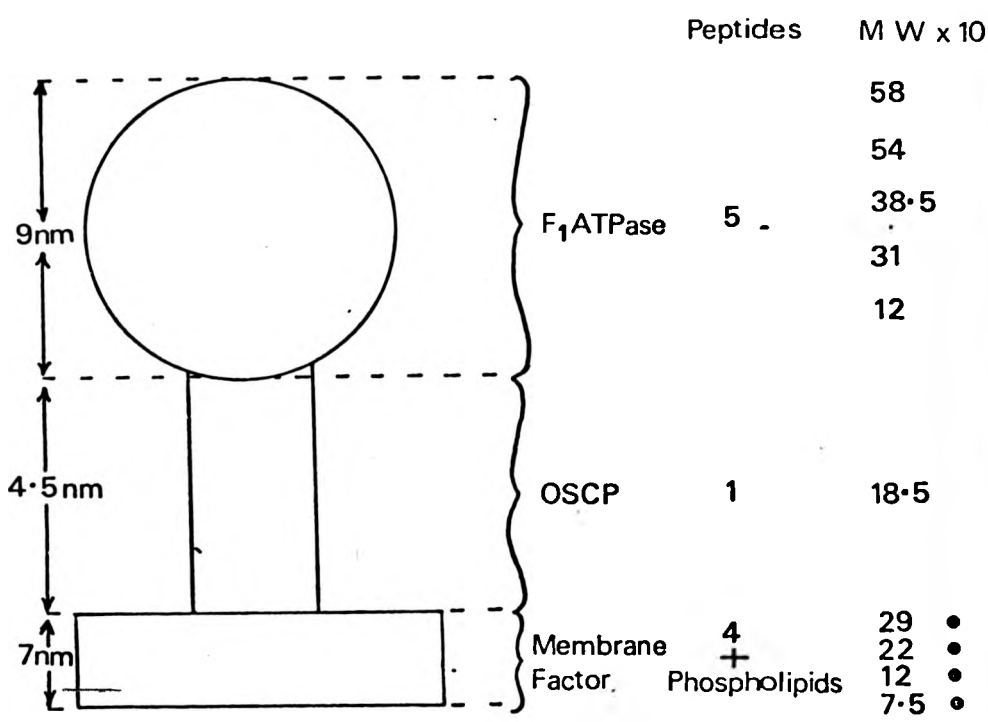
Membrane bound ATPase has been shown to be sensitive to a range of inhibitors (Griffiths and Houghton, 1974) but loses sensitivity to oligomycin, triethyl tin and venturicidin when detached from the membrane. More recently several procedures have been outlined which resulted in a solubilised ATPase preparation which displays sensitivity to oligomycin (Swanljung, 1973; Tzagoloff, 1969a; 1971). Whereas removal of the oligomycin insensitive $F_1$ ATPase from the membrane is usually accomplished by sonication the initial solubilization of the oligomycin sensitive enzyme is accomplished by treatment with disruptive agents such as cholate or deoxycholate or by non-

ionic detergents such as Triton X-100 (Tzagoloff et al., 1968b; Stekhoven et al., 1972; Capaldi, 1973; Swanljung et al., 1973). This solubilisation procedure appears necessary for the removal from the membrane of at least three highly insoluble lipophillic peptides necessary for the conferral of oligomycin sensitivity (Tzagoloff, 1973) and termed the membrane factor by Tzagoloff.

The preparation of Swanljung (Swanljung, 1973) begins with a Triton X-100 extract which is purified on an affinity column of Sepharose 4B covalently coupled to ATPase inhibitor protein or alternatively is purified by detergent gradient chromatography (Swanljung, 1973). However in these preparations the recovery of ATPase activity was low, 4.2% of total bovine heart mitochondrial activity being recovered as was the specific activity increasing from 2.1 moles Pi/min/mg in sub mitochondrial particles to 8.5 moles Pi/min/mg in the purified enzyme.

To date the most highly purified preparation is that of Tzagoloff and Meagher (1971) from the mitochondria of Saccharomyces cerevisiae. The purification scheme consists of solubilizing the enzyme from sub mitochondrial particles with Triton X-100, centrifuging once at high speed and sedimenting the supernatant on a glycerol gradient (5 to 15% w/v). The purified complex has a specific activity of 28 $\mu$moles Pi/min/mg and although the preparation still contains some phospholipid it appears under the electron microscope to be a fairly homogeneous complex of oval shaped globular particles 100 by 150A in size. Sedimentation analysis corrected for lipid content gives an upper limit for the molecular weight of 468,000 daltons. This preparation gave both a better recovery of

Figure 1.2




Schematic Representation of the Structure of Oligomycin Sensitive ATPase

• Mitoribosomal products (Tzagoloff and Meagher, 1972).

ATPase activity, some 22% of sub mitochondrial particle activity being recovered in the pure enzyme, and a higher specific activity, rising from 2.3 μmoles Pi/min/mg in sub mitochondrial particles to 28.0 μmoles Pi/min/mg in the purified enzyme, than the Swanljung preparation.

The rigorous purification and characterisation of the oligomycin sensitive ATPase complex from mammalian sources had not been achieved. Several preparations of low specific activity ( < 9 μmoles Pi/min/mg) have been obtained from bovine heart mitochondria by using cholate or deoxycholate and ammonium sulphate fractionation (Tzagoloff, 1973b; Capaldi, 1973). Although oligomycin and/or DCCD sensitive ATPase preparations have not been obtained as yet from bacterial or chloroplast membranes there is good reason to believe that such complexes exist. Similar to mitochondrial ATPase the membrane ATPase of chloroplasts and bacteria are sensitive to either oligomycin, DCCD or both (Vambutas and Racker, 1965; Harold et al., 1969).

## 1.6 SCOPE OF THIS THESIS

At the time when the work described in this thesis was begun some preliminary biochemical investigations had been carried out at the mitochondrial level on some mutants selected as resistant to oligomycin (Griffiths et al., 1972). There was, however, considerable controversy concerning the levels of ergosterol found in different mutants. Swanljung and co-workers claiming altered sterol levels in whole yeast, mitochondrial membranes and ATPase (Swanljung et al., 1972) while Griffiths and co-workers found no significant change in sterol levels of

OS ATPase from wild type and mutant cells (Griffiths and Houghton, 1974).

This then was taken as the starting point for work on these mutants. Chapter 2 is concerned with a preliminary investigation into the possible site for the factor(s) responsible for the conferral of the resistance phenomena of the membrane bound ATPase. The factor(s) were found to reside in the membrane portion of the enzyme complex. As described in the introduction to Chapter 2 E.S.R. studies were then used as an indirect approach to investigate any possible gross changes in membrane lipids. No discernible differences were found between mutant and wild type mitochondrial membranes.

On the basis of these findings it was decided to investigate any possible resistance phenomena at the level of a highly purified, inhibitor sensitive enzyme complex. Chapter 3 describes this work and the difficulties encountered in such an approach. The levels of resistance in the purified enzyme of both wild type and mutant cells correlated well with those found in SMP, however no physical changes in the enzyme during purification or on analysis with polyacryamide gels could be found to account for the resistance phenomena.

It was then decided to monitor the biogenesis of enzyme sensitivity in a system which could identify possible aberrant mitoribosomal products. Such an investigation is described in Chapter 4. Although some anomalies were found during the biogenesis of inhibitor sensitive ATPase in mutant A21 it was not possible to positively identify a peptide whose temporal or gross production was responsible for the resistance phenomenon.

It appeared that a more direct unambiguous approach should be tried and that this would involve purification and subsequent analysis of the four mitoribosomal products associated with the OS ATPase (Tzagoloff et al., 1973). The observation that neutral chloroform : methanol (2 : 1 v/v) extracted a single mitoribosomally produced proteolipid, which also appeared to be subunit 9 of the OS ATPase coupled with the identification by Beechey and co-workers (Cattell et al., 1971) of a low molecular weight proteolipid as the binding site for DCCD, an oligomycin like inhibitor, in rat liver mitochondria, prompted an initial purification and characterisation of subunit 9. The insurmountable difficulties involved in attempts to further analyse this peptide from wild type and mutant cells are discussed in detail in Chapter 5.

# CHAPTER 2

## RECONSTITUTION OF OLIGOMYCIN SENSITIVE ATPase AND ELECTRON SPIN RESONANCE STUDIES

### 2.1 INTRODUCTION

A series of mutants of Saccharomyces cerevisiae have been selected on the criteria of their ability to grow on glycerol in the presence of the inhibitor of oxidative phosphorylation, oligomycin. The mutants selected for use in this study displayed cytoplasmic inheritance and mapped at three loci on the mitochondrial genome: 1) OL1 represented by D22A21 in this study 11) OL11 represented by D22A15 111) OL111 represented by D22A19. Loci OL1 and OL111 map very closely together and probably manifest themselves in the same translation product. Loci OL1 and OL11 are widely separated on the mitochondrial genome and probably give rise to two different peptides. Studies at the level of mitochondria and sub mitochondrial particles indicate that the level of resistance to oligomycin in these mutations increases in the order A15<A21<A19 (Griffiths and Houghton, 1974).

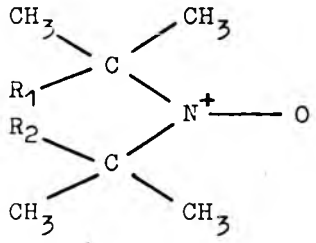
The mitochondrial ATPase complex can be fractionated into three components: 1) $F_1$ ATPase 11) Oligomycin sensitivity conferring protein and 111) a membrane fraction responsible for oligomycin sensitivity. By fractionating and reconstituting the membrane bound complex utilizing various components from different strains it should be possible to identify that fraction of the ATPase complex which contains the factor(s) responsible for the change in oligomycin sensitivity. The first part of this chapter describes such a study.

The reconstitutions of membrane bound oligomycin sensitive ATPase described in this chapter were carried out using $F_1$ ATPase and OSCP from bakers yeast. Both of these components of the oligomycin sensitive ATPase are nuclear coded and synthesised by the cytoribosomal system (Tzagoloff et al., 1973) and as such would be unaltered in a cytoplasmically inherited mutant. The mitochondrially synthesised and therefore probably coded peptides of the oligomycin sensitive ATPase are those lipophilic peptides which constitute the membrane factor(s) (Tzagoloff et al., 1973). Any cytoplasmically inherited mutation which affects oligomycin sensitivity should presumably manifest itself in these membrane factors. To this end $F_1$ ATPase and OSCP depleted membranes were prepared from wild type and mutant strains and their membrane bound, oligomycin sensitive ATPase activity restored using $F_1$ ATPase and OSCP from a common source (bakers yeast) and the resulting activity investigated to see if the resistance phenomena present in the original sub mitochondrial particles was still present. If this was so then the cytoplasmically inherited resistance factor(s) must lie in the membrane factor of the oligomycin sensitive ATPase and not in the $F_1$ ATPase or the OSCP.

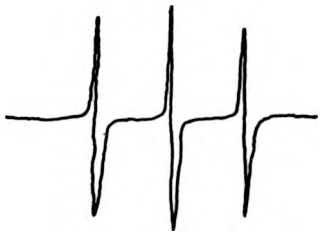
The second part of this chapter is concerned with investigating possible gross changes in mitochondrial membrane composition in cytoplasmically inherited oligomycin resistant mutants. This study was prompted by controversy over possible correlation of oligomycin resistance with altered sterol levels of whole yeast cells, mitochondrial membranes and oligomycin sensitive ATPase (Swanljung et al., 1972; Griffiths and Houghton, 1974). Because of variable levels of sterols reported

for such mutants and because sterol levels in inner mitochondrial membranes are low (Parsons and Yano, 1967) it was decided to use a different indirect approach to look for possible changes in mitochondrial membrane composition. This approach consisted of 1) E.S.R. studies of purified mitochondrial membrane fluidity in wild type and mutant strains, 11) titration of $^{3}H$ NEM against mitochondrial membranes of wild type and mutant strains to determine whether any gross alterations in the stereochemistry of the membrane had taken place altering the availability of titratable protein sulphydryl groups.

Spin labelling studies employ nitroxides with the following general structure:-




The param agnetic properties of these compounds derive from an unpaired electron localised primarily on the nitrogen. Spectra obtained with free tumbling nitroxides characteristically have three equally spaced components as shown below.



The magnetic field experienced by the unpaired electron is perturbed by the nuclear magnetic moment of $^{14}N$, this having a spin quantum number of 1 allows only three orientations, parallel, antiparallel and perpendicular, of the nuclear magnetic moment with respect to the laboratory field. These three perturbing fields split the hypothetical single electron resonance band into three equally spaced components.

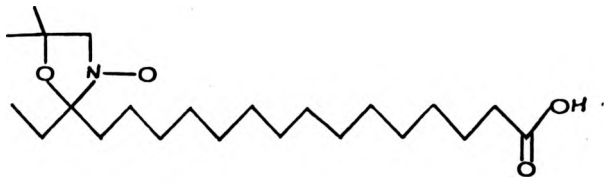
In the first spin labelling study of oriented phospholipid multilayers (Libertini et al., 1969) spin labels were used to examine the overall orientation of the lipid chains in the multilayers. Nitroxide free radicals attached to stearic acid were shown to be orientedin the egg lecithin multilayers and the results indicated that the spin labels are not sufficiently large perturbations to cause gross local disorder. The spin labels employed in this study were the 4',4'-dimethyloxazolidine-N-oxyl derivative of methylketostearate which for simplicity is referred to as the doxyl moiety ie. 5-doxylstearic acid and 16-doxylstearic acid. In these derivatives the doxyl moiety is rigidly bonded through a spirane structure to the flexible backbone of the stearic acid moiety, thus the nitroxide has no movement independant of the stearic acid and so its movement reflects the movement of the whole molecule.

The work of Jost et al., (1971) showed that with such probes the splitting of the spectra obtained depended on the position of the doxyl group. The splitting decreases markedly as the doxyl group is moved away from the carboxyl end, ie. towards the centre of the phospholipid bilayer. The qualitat ive interpretation of this is that molecular movement increases markedly towards the hydrocarbon end of the fatty acid.chains.

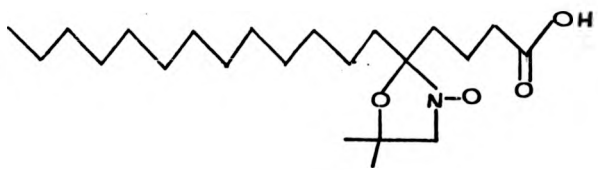
The further along the fatty acid chain the doxyl group is situated the more similar the spectrum becomes to that of a free tumbling nitroxide (Mehlhorn and Keith, 1972).

It has been widely reported that the addition of sterols to lipid bilayers reduces the fluidity of the bilayers (Shimshick and McConnell, 1973; Cogan et al., 1973; Hubbel and McConnell, 1971; Oldfield et al., 1971; Hsia et al., 1972). Thus any gross changes in sterol composition of the mitochondrial membrane should reflect itself in altered membrane fluidity which should be apparent in a changed E.S.R. spectrum showing greater or lesser splitting.

The E.S.R. probes chosen for this study were two nitroxide labelled stearic acid derivatives. The first, 16 doxyl stearic acid, has its nitroxide moiety bound at the sixteenth carbon atom from the carboxyl end, ie. very close to the hydrocarbon end of the stearic acid molecule.




The second E.S.R. probe uses 5 doxyl stearic acid.



As a preliminary experiment to any possible E.S.R. studies with N-ethylmaleimide spin labels it was decided to titrate $^{3}$H-N-ethylmaleimide against the wild type and mutant membranes for the reasons discussed earlier. No discernible differences were found in titratable levels of N-ethylmaleimide and so in view of the expense involved in the purchase of nitroxide labelled N-ethylmaleimide this experiment was discontinued.

## 2.2 MATERIALS AND METHODS

### Yeast Strain

The haploid strain used routinely on this investigation for the isolation of mutants was D22 a $ad^{-}_{2}$ $arg^{+}$ $met^{+}$ $\rho^{+}$ $\omega^{+}$ $C^{S}$ $Er^{S}$ $OL^{S}$ $Sp^{S}$ $Cy^{S}$ $CP^{S}$. This strain was the gift of Dr. D. Wilkie. The mutants were produced using ultraviolet light as the mutagen and selected as described by Avner and Griffiths (1970; 1973).

### Growth of Saccharomyces cerevisiae

S. cerevisiae was grown aerobically in 10 litre cultures in New Brunswick fermentors (New Brunswick Scientific Co., Imc., New Brunswick, New Jersey, U.S.A.) in the dark at 30°C. Compressed air was passed through the culture at the rate of 1 lit. air/min/lit. culture. The culture was stirred at a rate of 450r.p.m. The medium contained per litre of distilled water:- 1 g $KH_2PO_4$, 1.2 g $(NH_4)_2SO_4$, 0.5 g NaCl, 0.7 g $MgSO_4.7H_2O$, 0.4 g $CaCl_2$, 0.3% yeast extract, 0.2 ml tributyl citrate antifoaming agent and in cultures of haploid D22 0.1 g adenine sulphate. Glucose at a concentration of 0.8% was used as a carbon source and was added as a sterile 40% solution

after autoclaving. The culture was inoculated with a 1% v/v starter culture grown to stationary phase on 0.8% glucose.

Preparation of Asolectin

Purified soya bean asolectin was thoroughly homogenised in 10 mM Tris, 1 mM EDTA at a concentration of 20 mg/ml. The mixture was then sonicated for three one minute bursts with cooling on ice between sonications (M.S.E. 50 KHz sonicator, maximum power). The mixture was then spun for 100,000g for ten minutes. The supernatant was stored frozen in 5 ml aliquots. The phospholipid content was determined by weighing to constancy to be 11.2 mg/ml.

Preparation of Sub Mitochondrial Particles.

Fermenter grown cells were harvested in early stationary phase by centrifugation in an M.S.E. "Mistral" centrifuge (6 x 1 lit. rotor, 2,000 r.p.m. for 5 minutes). This and all subsequent steps were carried out at 0 to 4°C. The cells were washed once with distilled water and resuspended in ice cold buffer containing : 0.25 M mannitol, 1 mM EDTA, 20 mM Tris acetate pH 7.5. 10 g wet weight of cells were suspended in 30 ml buffer. The cells were homogenised using a Braun shaker (B. Braun Apparatebau, Melsungen, Germany) with 0.45 to 0.5 mm diameter glass beads at top speed (4,000 r.p.m.) for 45 sec. One part by volume of cell suspension was homogenised with one part by weight of glass beads (24 ml of beads).

The homogenate was decanted and the beads washed twice with 10 ml portions of buffer. Homogenate and washings were pooled and centrifuged in a G.S.A. rotor using a Sorvall RCB-2 centrifuge at 4,000 r.p.m. for 15 minutes. The supernatant was

then spun at 15,000 r.p.m. for 20 minutes in an SS 34 rotor. The pellet was homogenised in buffer containing 0.25 M sucrose, 10 mM Tris acetate pH 7.5 and spun at 4,500 r.p.m. for 20 minutes in an SS 34 rotor. The supernatant was then spun at 15,000 r.p.m for 20 minutes in an SS 34 rotor. The pellet was homogenised in 0.25 M sucrose, 10 mM Tris acetate pH 7.5 to give a final protein concentration of 25 to 40 mg/ml.

The mitochondrial suspension was sonicated for 30 sec., on ice, in an M.S.E. 150 K sonic oscillator at the medium setting, and then centrifuged at 40,000 r.p.m. for 30 minutes in a Beckman 40 rotor in a Beckman L2-50 ultracentrifuge. The lipid layer and supernatant were aspirated off, the pellet was resuspended in 0.25 M sucrose, 10 mM Tris acetate pH 7.5 to a final protein concentration of 25 mg/ml.

Protein Estimations

Protein estimations were by the method of Lowry et al., (1951). The assay was modified in that after addition of the phenolic reagent the tubes were placed in a boiling water bath for exactly one minute, cooled on ice and the optical density determined. This procedure considerably shortened the assay time giving the same results as the traditional method. When assaying particulate preparations 0.1 ml of 1% SDS was included in the assay as a solubilising agent.

Preparation of Sub Mitochondrial Particles from Bakers Yeast

The method employed for the large scale production of sub mitochondrial particles from bakers yeast was essentially that outlined by Tzagoloff (1969) and consisted of crumbling two pounds of pressed yeast into a one gallon Dewar flask containing about 3 litres of liquid nitrogen, the excess

nitrogen was decanted off for reuse and the frozen pellets transferred to a one gallon stainless steel Waring Blendor container (Eberbach Corporation, Michigan). The blendor was immediately switched on at low speed, any delay at this point results in frozen and immobilised blendor bearings, and run until the pellets were converted into a fine swirling powder. The powder was blended for a total of three minutes in three equal bursts, the powder being redistributed with a spatula between bursts. The powder was then transferred to 1.5 litres of buffer maintained at room temperature and containing 0.4 M sucrose, 0.05 M Tris acetate pH 8.2 and 1 mM EDTA. After the powder had thawed the pH was adjusted to 7.5 with NaOH and the suspension was homogenized at low speeds for 45 secs in the Waring Blendor. The homogenate was centrifuged for 15 minutes at 2,000 r.p.m. in an M.S.E. "Mistral" centrifuge equiped with a 6 x 1 lit. head. The supernatant was then spun at 10,000 r.p.m. for 40 minutes in a G.S.A. head in a Sorvall RC 2B centrifuge. The pellet was resuspended in 500 ml of buffer containing 0.25 M sucrose and 0.01 M Tris acetate pH 7.5. After homogenisation in a glass-Teflon homogeniser the suspension was centrifuged at 4,000 r.p.m. for 15 minutes in a Sorvall G.S.A. head. The supernatant was spun at 10,000 r.p.m. for 40 minutes in a Sorvall G.S.A. rotor. The pellet was resuspended in about 150 ml of 0.25 M sucrose, 0.01 M Tris acetate pH 7.5 and homogenised in a glass-Teflon homogeniser to give a final protein concentration of between 25 to 40 mg/ml. The suspension was sonically irradiated in 50 ml batches for 30 sec. in an M.S.E. 150 Kc. sonic oscillator at medium power output, the suspension was cooled on ice during sonication. The suspension

was centrifuged for 40 minutes at 40,000 r.p.m. in a Beckman 40 head. The supernatant was aspirated off and the pellet was resuspended in 50 ml of buffer containing 0.25 M sucrose, 0.01 M Tris acetate pH 7.5 and recentrifuged at 40,000 r.p.m. for 40 minutes in a Beckman 40 rotor. The washed particles were resuspended in the sucrose-Tris buffer and the protein concentration adjusted to 25 mg/ml.

Preparation of $F_1$ ATPase from Bakers Yeast Sub Mitochondrial Particles

The procedure used for the preparation of yeast $F_1$ ATPase was a modified version of that described by Tzagoloff (1969a). Bakers yeast sub mitochondrial particles at a concentration of 25 mg/ml in 0.25 M sucrose, 0.01 M Tris acetate pH 7.5 were diluted to 12.5 mg/ml with 10 mM Tris acetate pH 7.5. ATP was added to a final concentration of 2 mM, EDTA was added to a final concentration of 1 mM and the suspension brought to pH 8.5 with Tris base.

The suspension was sonicated in an M.S.E. 150 Kc. sonic oscillator in 50 ml batches without cooling at maximum power. Sonication was continued until the temperature of the sample rose to 35°C, usually 2.5 to 3 min. All subsequent steps were carried out at room temperature. The pooled particle suspension was adjusted to pH 5.8 with 1 N acetic acid and centrifuged at 30,000 r.p.m. for 20 minutes in a Beckman 30 rotor. The pH of the supernatnat was adjusted to 7.5 with 1 M Tris base.

Solid ammonium sulphate was slowly added to 50% saturation. The suspension was spun at 30,000 r.p.m. for 20 minutes. Solid ammonium sulphate was added to the supernatant to bring the degree of saturation to 75%. The suspension was again spun at

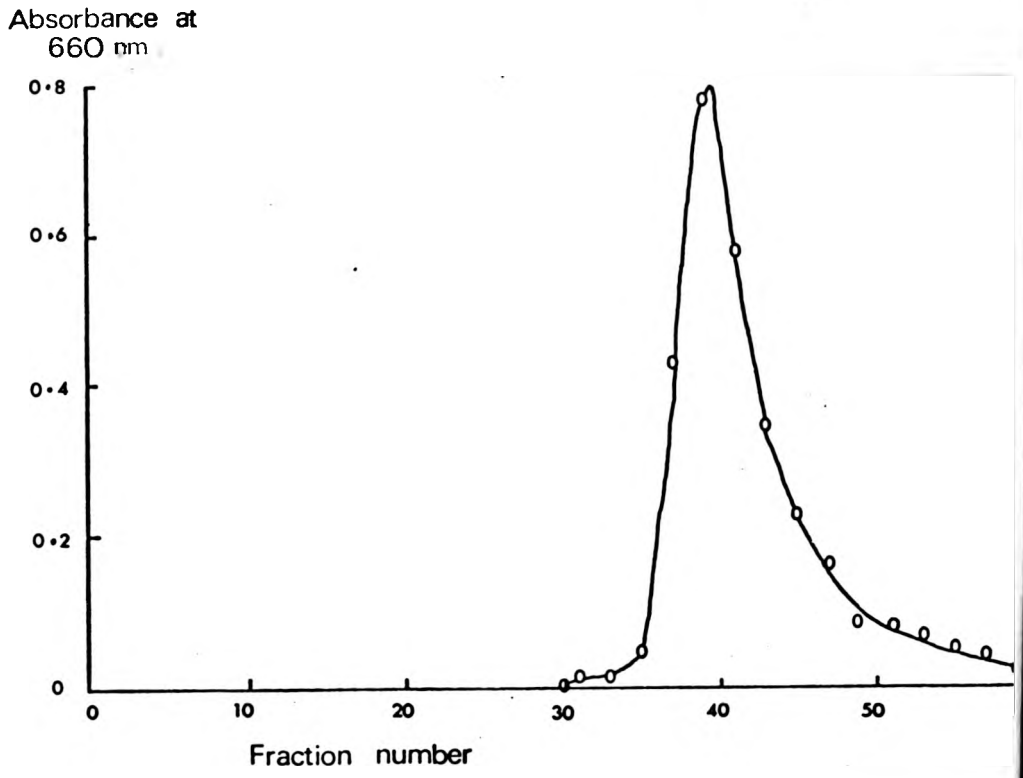
30,000 r.p.m. for 20 min. and the supernatant discarded. The pellet was resuspended in a small volume of buffer containing 10 mM Tris acetate pH 8.0, 2 mM ATP and 1 mM EDTA hereafter referred to as TEA. This sample was then applied to a Sephadex G-50 column (3 cm x 15 cm) equilibrated with TEA. The coloured part of the eluate was collected and diluted approximately ten fold with TEA.

The whole sample was then applied to a column of DEAE cellulose (Whatman pre-swollen D.E. 52, 2 cm x 8 cm) equilibrated with TEA. The column was washed with:-

1) 20 ml of TEA
2) 70 ml of TEA containing 50 mM KCl
3) TEA containing 300 mM KCl.

With application of the third wash containing 300 mM KCl the coloured band moved through the DEAE cellulose and was collected. The eluate containing the coloured band was brought to 75% saturation with the careful addition of solid ammonium sulphate. The suspension was spun at 30,000 r.p.m. for 20 min in a Beckman 30 rotor. The pellet was resuspended in a small volume of TEA and applied to a Biogel A5 column (3.2 cm x 63 cm) equilibrated with TEA. The column was developed with TEA and approximately 5 ml fractions were taken. Alternate fractions were assayed for ATPase activity and the active fractions (see Fig. 2.1m) pooled. Solid ammonium sulphate was added to 75% saturation and the enzyme could be stored at $4^{o}C$ without loss of activity. The yield was approximately 9 mg of enzyme from 8.0 g of submitochondrial particles and the specific activity was between 18 and 23 $\mu$moles Pi/min/mg.

Figure 2-1m




Elution Profile of $F_1$ ATPase from a Biogel A-5 Column

The column was developed as described in Materials and Methods. 10 $\mu$l of each fraction was assayed for ATPase activity for 12 minutes at 37°C pH 8.5. The activity of each fraction is expressed as the absorbance at 660nm produced in the assay for hydrolysed phosphate.

Preparation of Oligomycin Sensitivity Conferring Protein from Bakers Yeast Sub Mitochondrial Particles

Oligomycin sensitivity conferring protein was prepared by a modified method of MacLennan and Tzagoloff (1968) using sub mitochondrial particles as the starting material rather than oligomycin sensitive ATPase and omitting fractionation on CM-cellulose.

Yeast submitochondrial particles at 25 mg/ml in 0.25 M sucrose, 0.01 M Tris acetate pH 7.5 were diluted to 18 mg/ml with 10 mM Tris acetate pH 7.7. 0.5 ml volumes of 1 N $NH_4OH$ was added slowly with stirring, the suspension was incubated at 0°C for 10 min. 2 N acetic acid was added until the pH was brought to 7.5 and the suspension was centrifuged at 30,000 r.p.m. for 20 min in a Beckman 30 rotor.

The pH of the supernatant was brought to 5.0 with 2 N acetic acid and the resulting suspension centrifuged at 30,000 r.p.m. for 20 min as before. Solid ammonium sulphate was added to the clear supernatant to bring the solution to 60% saturation. The suspension was spun at 30,000 r.p.m. for 10 min and the pellet resuspended in 70% saturated ammonium sulphate and stored at 4°C. Between 4.5 and 7.5% of the sub mitochondrial particle protein was recovered.

Preparation of Oligomycin Sensitivity Conferring Protein depleted and $F_1$ ATPase Depleted Particles

Particles depleted of $F_1$ ATPase and OSCP, hereafter referred to as $NH_4Br$ particles, were prepared from wild type and mutant sub-mitochondrial particles by a modification of the procedure of Tzagoloff (1969a).

Sub mitochondrial particles at a protein concentration of

25 mg/ml were diluted to 8 mg/ml with 0.25 M sucrose, 10 mM Tris acetate pH 7.5. To this suspension was added an equal volume of 6 M NaBr and the suspension was centrifuged at 40,000 r.p.m. for 30 min in a Beckman 40 rotor. The infranatant was carefully removed with a Pasteur pipette and the large floating layer and small pellet were resuspended inthe original volume of 0.25 M sucrose, 10 mM Tris acetate pH 7.5. To this suspension was added half a volume of 1 N $NH_4OH$ and the mixture was incubated at 0°C for 10 min. To this was added a quarter of the original volume of 1 N acetic acid, the resulting suspension was centrifuged at 40,000 r.p.m. for 30 min in a Beckman 40 rotor. The pellets were resuspended in the original volume of 0.25 M sucrose, 10 mM Tris acetate pH 7.5 and half a volume of 1 N $NH_4OH$ was added and the suspension was incubated at 0°C for 10 min, then a quarter of the original volume of 1 N acetic acid was added and the suspension spun at 40,000r.p.m. for 30 min in a Beckman 40 rotor. The pellets were washed with the original volume of 0.25 M sucrose, 10 mM Tris acetate pH 7.5 and resuspended in a small volume of the same. Generally 20 to 26% of the sub mitochondrial protein was recovered.

Reconstitution of Oligomycin Sensitive ATPase.

Bakers yeast $F_1$ ATPase and bakers yeast OSCP were centrifuged out of the ammonium sulphate suspensions and resuspended in 0.25 M sucrose, 10 mM Tris acetate pH 7.5 and the protein concentration adjusted to 2 mg/ml and 10 mg/ml respectively. The $NH_4Br$ extracted particles from wild type and mutant strains was adjusted to 5 mg/ml. The various fractions were mixed as shown in Table 2.1 in a final volume of 1 ml. The mixtures

were incubated for 10 min at 30°C, the mixture was then diluted with two volumes of cold buffer containing 0.25 M sucrose, 10 mM Tris acetate and centrifuged at 15,000 r.p.m. for 10 min in a Sorvall RC 2B centrifuge using an SS 34 rotor. The precipitate was resuspended at an $NH_4Br$ extracted particle concentration of 1 mg/ml and assayed for ATPase activity. All specific activities were calculated on the basis of the amount of $NH_4Br$ particles present in the assay. For inhibitor studies 4 mg of $NH_4Br$ extracted particles, 1.6 mg $F_1$ ATPase and 16 mg OSCP were incubated for 10 min at 30°Cin a final volume of 8 ml. Two volumes of cold buffer were added and the mixture centrifuged at 15,000 r.p.m. for 10 min as before. The pellet was homogenised in a glass-Teflon homogeniser and brought to a final volume of 4 ml in 0.25 M sucrose, 10 mM Tris acetate pH 7.5. The equivalent of 200 µg of $NH_4Br$ extracted membranes were used to assay for ATPase activity.

Assay of ATPase Activity

ATPase activity was measured in a reaction mixture containing 5 mM ATP, 2 mM $MgCl_2$, 50 mM Tris HCl pH 9.5 in a final volume of 1 ml. Enzyme and inhibitor were preincubated together for 5 min at 30°C and the reaction initiated by the addition of 50 µl of 0.1 M ATP brought to pH 7.5 with NaOH. The reaction was terminated by the addition of 1 ml of cold, 10% w/v trichloracetic acid. The mixture was centrifuged at 6,000 r.p.m. for 5 min to sediment the protein and 0.5 ml samples of the supernatant were removed for the estimation of phosphate by the method of King (1932). Inhibitors were added as an ethanolic solution which never exceeded 1% of the final assay volume and had no deleterious effect on enzyme activity over

the time scales used (see Fig. 3.1m).

Preparation of Mitochondria for E.S.R. and $^{3}H$ -NEM Binding Studies

Cells were grown, harvested and broken in the Braun shaker as described. 5 ml of the washed mitochondrial fraction at a protein concentration of 25 - 30 mg/ml was layered onto a discontinuous gradient consisting of 12.5 ml of each of 70, 50, 30 and 15% sucrose in 10 ml Tris acetate, 1 mM EDTA pH 7.5. The gradients were spun for 2.5 hours at 23,000 r.p.m. in a Beckman 25.2 SW rotor. The dark coloured cytochrome laden band was removed with a Pasteur pipette and diluted four fold with 10 mM Tris acetate pH 7.5 and spun at 15,000 r.p.m. for 20 min in a Sorvall SS 34 rotor. The pellet was homogenised in a glass-Teflon homogeniser in 5 ml of 0.25 M sucrose 10mM Tris acetate pH 7.5 and layered onto a second gradient constructed in the same fashion as the first and the whole procedure repeated. The final mitochondrial pellet was resuspended in 0.25 M sucrose, 10mM Tris acetate pH 7.5 at a protein concentration of 10 mg/ml.

Labelling of Mitochondria with $^{3}H$-NEM

N-ethyl 2-$^{3}H$-maleimide was supplied by New England Nuclear Inc. at an activity of 0.25 mCi in 0.14 mg in 0.25 ml pentane. Pentane was considered too volatile a solvent so the sample was dried down and redissolved in 0.25 ml of ethanol. The method of labelling was a combination of those described by Green et al., (1973) and Hatase et al., (1973). 1 mg of mitochondrial suspension in 3 mM $MgCl_2$ was mixed with the indicated amounts of $^{3}H$-NEM and incubated at $0^{o}C$ for 20 min. The mitochondria were sedimented at 100,000 g for 30 minutes, washed and resedim-

ented. The surface of the pellet was washed three times with cold 0.25 M sucrose, 10 mM Tris acetate pH 7.5. The pellet was homogenised in 0.5 ml of the same solution and solubilised in 10 ml Triton, toluene, butyl PBD scintillant and counted.

## 2.3 RESULTS

### Effect of Oligomycin Sensitivity Conferring Protein on Membrane Bound ATPase Activity

From Table 2.1 it can be seen that only residual membrane bound ATPase activity is present in the sedimented $NH_4Br$ particles when either OSCP or $F_1$ ATPase is omitted from the reconstitution mixture and that all three components are necessary to reconstitute membrane bound ATPase activity. Table 2.1 also shows that the amount of ATPase activity restored to the $NH_4Br$ particles is clearly dependant upon the amount of OSCP in the reconstitution mixture.

The results depicted in Table 2.2 show that the inclusion of 5 mM $MgCl_2$ in the reconstitution mixture increases ATPase binding by some 39%, the further addition of 200 µg and 500 µg of asolectin per 500 µg of $NH_4Br$ particles in the presence of 5 mM $Mg^{++}$ increases the membrane bound activity by 53 and 74% respectively. Because of likely lipid effects upon enzyme inhibitor sensitivity (see Chapter 3, Figs. 3.8, 3.9, 3.10) (Tzagoloff, 1969a) asolectin was not included in the reconstitution mixture for inhibitor studies.

From Table 2.3 it can be seen that in no instance was it possible to reconstitute a membrane bound ATPase with a specific activity equivalent to that found in the original sub mitochondrial particles. That this may be due to mixing of

TABLE 2.1

Effect of OSCP on Reconstitution of membrane bound ATPase activity

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| $F_1$ ATPase $\mu$gm | 200 | - | 200 | 200 | 200 | 200 | 200 |
| $NH_4Br$ extracted particles $\mu$gm | 500 | 500 | 500 | 500 | 500 | 500 | 500 |
| OSCP $\mu$gm | - | 2000 | 100 | 200 | 500 | 1000 | 2000 |
| Specific Activity ($\mu$moles $PO_4$/ min/mg) | 0.252 | 0.19 | 0.228 | 0.258 | 0.312 | 0.492 | 1.05 |

TABLE 2.2

Effect of $Mg^{2+}$ and Phospholipids on Reconstitution of membrane bound ATPase activity.

| | | | | |
|---|---|---|---|---|
| $F_1$ ATPase µgm | 200 | 200 | 200 | 200 |
| $NH_4Br$ Extracted Particles µgm | 500 | 500 | 500 | 500 |
| OSCP µgm | 2000 | 2000 | 2000 | 200 |
| $Mg^{2+}$ mM | - | 5 | 5 | 5 |
| Phospholipid µgm | - | - | 200 | 500 |
| Specific Activity (µmoles $PO_4$/min/mg) | 0.93 | 1,3 | 1,43 | 1.61 |

TABLE 2.3

Specific Activities of Sub Mitochondrial Particles and Reconstituted ATPase

| Strain | D22 | A21 | A19 | A15 |
|---|---|---|---|---|
| Sub Mitochondrial Particles | 4.7 | 4.82 | 4.13 | 5.3 |
| Reconstituted Particles | 1.32 | 1.05 | 0.87 | 1.5 |

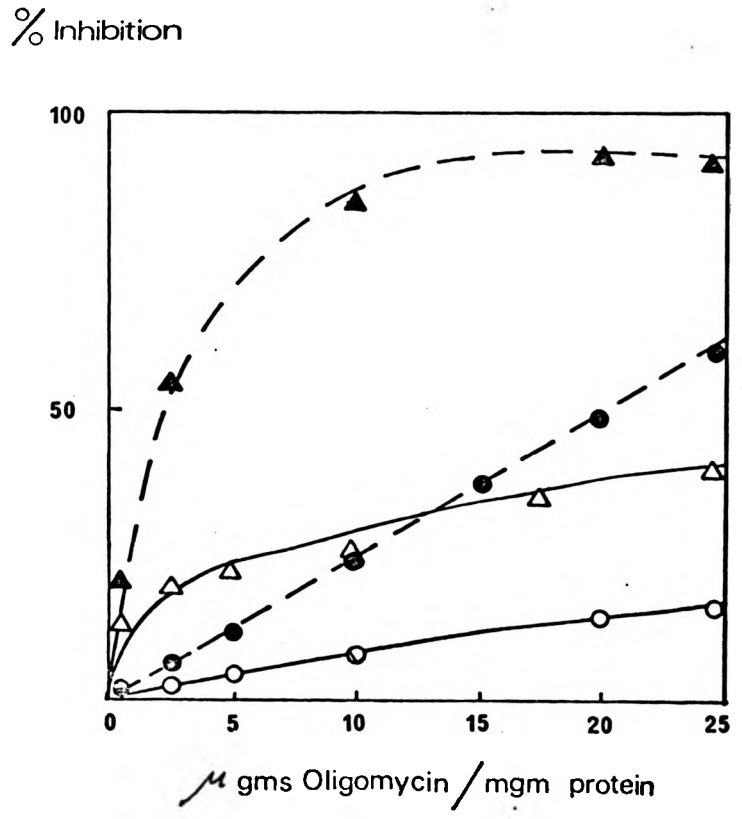
The numbers express specific activities as $\mu$moles Pi/min/mg protein. Reconstituted particle specific activities are calculated on the basis of the amount of $NH_4Br$ particles present in the assay.

components of ATPase from different strains was discounted as on reconstituting all three components derived from wild type sub mitochondrial particles the specific activity of the reconstituted particles was not significantly different from the specific activities of hybrid reconstituted particles.

From Figs. 2.1, 2.2, and 2.3 it can be seen that the level of sensitivity and final levels of inhibition of the reconstituted membrane bound ATPases is significantly lower than in the equivalent sub mitochondrial particles both for the wild type and mutant strains. This may be due to either:-
1) Incomplete removal of $F_1$ ATPase during sedimentation leaving a residue of highly active oligomycin insensitive activity. This, however, seems unlikely as column 1 of Table 2.1 shows that no significant amount of unbound $F_1$ ATPase activity is sedimented. 2) Some ATPase may be incorrectly reconstituted onto the membrane resulting in ATPase activity without oligomycin sensitivity, this possibility cannot be discounted. However from Figs. 2.1, 2.2 and 2.3 it is clear that a distinct differential in oligomycin sensitivity is found between the wild type and mutant reconstituted membranes.

Fig. 2.4 shows the E.S.R. signal obtained when the E.S.R. probe C16 doxyl stearic acid is incorporated into the mitochondrial membranes of wild type D22 and mutants A21, A19 and A15. Qualitatively no difference can be seen in the spectra, a quantitative analysis of the ratio of peak heights $h_o/h-1$ shows no significant differences. On this probe the nitroxide moiety will be orientated deep in the membrane phospholipid bilayer where the hydrocarbon chain will rotate relatively freely, thus there would appear to be no significant differences

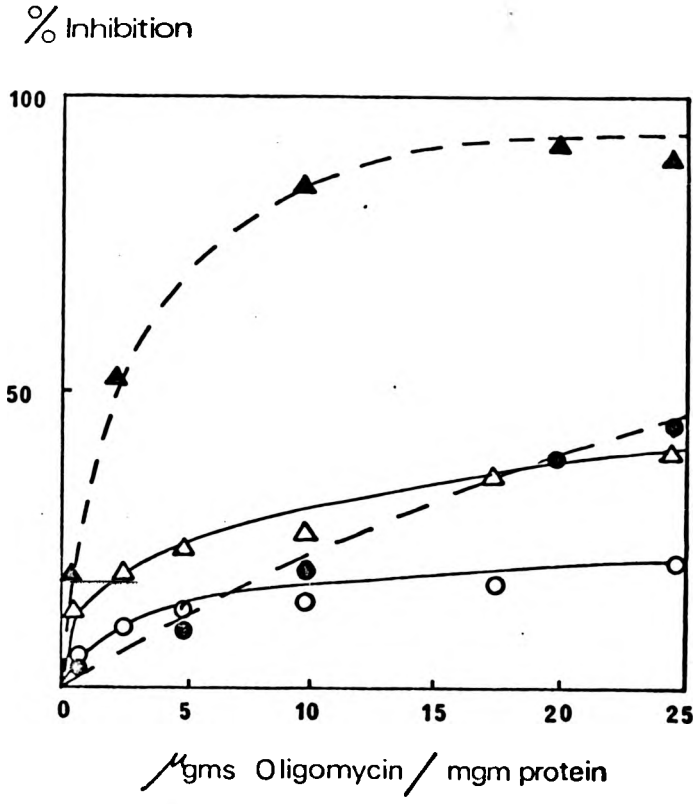
Fig. 2.1




Reconstitution of D22 and A21 OS ATPase from $F_1$ ATPase, OSCP and Membrane Fraction

The reconstitutions were carried out as described in Materials and Methods. The sensitivity of SMP from D22 ( ▲--▲ ) and A21 ( ●--● ) are as indicated. The reconstituted OS ATPase sensitivities using bakers yeast $F_1$ ATPase and OSCP and membrane fraction from D22 ( Δ—Δ ) and A21 ( O—O ) were as indicated.

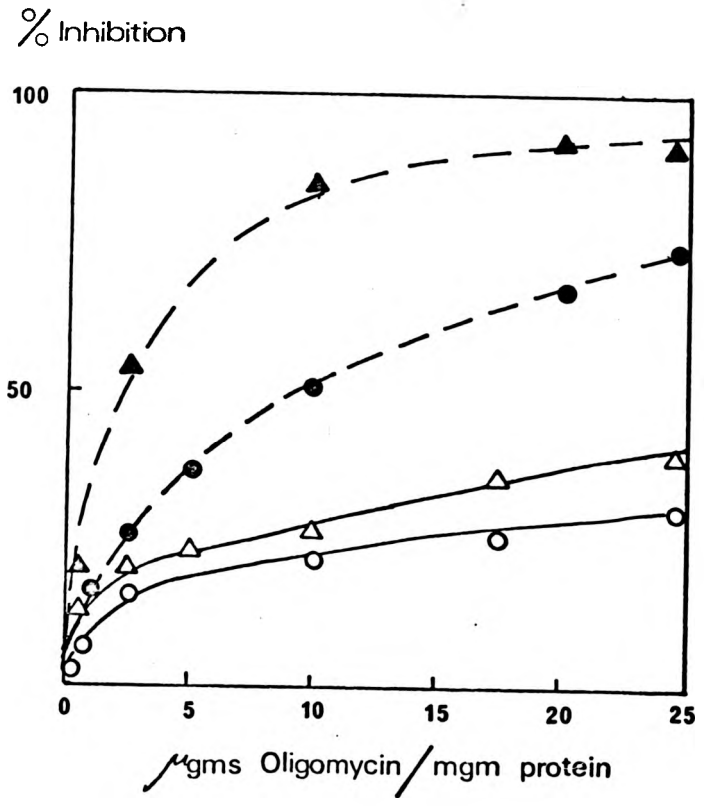
Fig. 2-2




Reconstitution of OS ATPase from $F_1$ ATPase, OSCP and Membrane Fraction

Reconstitution of OS ATPase activity was carried out as described in Materials and Methods. The sensitivity of SMP from D22 ( ▲--▲ ) and A19 ( ●--● ) were as indicated. The OS ATPase sensitivities reconstituted from bakers yeast $F_1$ ATPase and OSCP, and membrane fraction from D22 ( Δ—Δ ) and A19 ( O—O ) were as indicated.

Fig. 2.3




Reconstitution of OS ATPase from $F_1$ ATPase, OSCP and Membrane Fraction

Reconstitution of OS ATPase activity was carried out as described in Materials and Methods. The sensitivity of SMP from D22 (▲—▲) and A15 (●—●) were as indicated. The OS ATPase sensitivities reconstituted from bakers yeast $F_1$ ATPase and OSCP, and membrane fraction from D22 (Δ—Δ) and A15 (O—O) were as indicated.

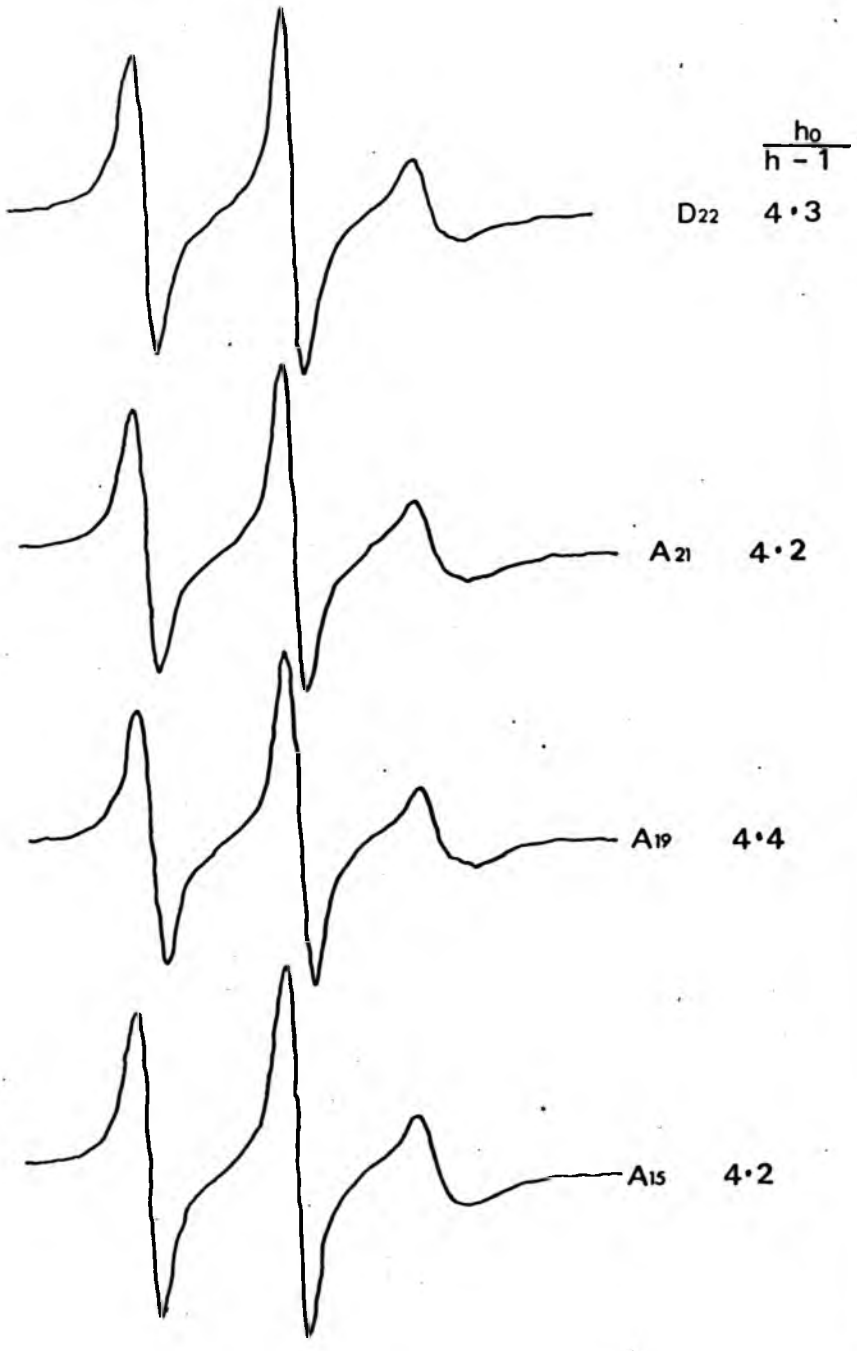



FIGURE 2.4

Mitochondrial membrane, at a protein concentration of 30mg/ml, were incubated with 16 doxyl stearic acid for five minutes at 0°C. The spectra were obtained by Dr. E. Bertoli.

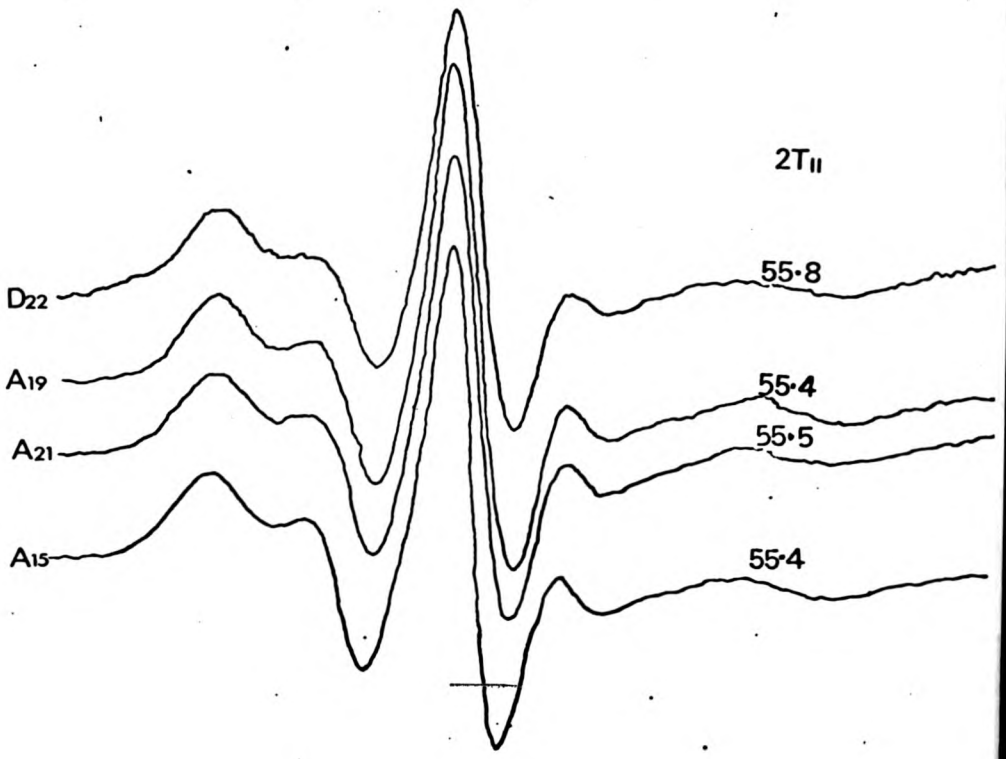



FIGURE 2.5

Mitochondrial membranes at a protein concentration of 30 mg/ml, were incubated with 5 doxyl stearic acid for five minutes at 0°C. The spectra were obtained by Dr. E. Bertoli.

in the fluidity of the mitochondrial membrane, deep within the membrane, between the wild type and any of the mutants investigated. Fig. 2.5 shows the E.S.R. signal obtained when the probe C5 doxyl stearate is incorporated into the wild type and mutant mitochondrial membrane. This probe has the nitroxide moiety situated near the carboxyl end of the molecule and so is much more restricted in its movements, however the results obtained with this probe lead to the same conclusions as those obtained with C16 doxyl stearate.

Titration of Wild Type and Mutant Mitochondrial Membranes with $^{3}$H N-Ethylmaleimide

Table 2.4 presents the results obtained when varied amounts of $^{3}$H-NEM are incubated with a fixed amount of membranes. No large discrepancies in the number of table sulphydryl groups between mutant and wild type strains although in all cases the amount of $^{3}$H-NEM bound per mg protein is lower than that reported by Green et al., (1973) and Hatase et al., (1970) for beef heart mitochondria.

## 2.4 DISCUSSION

It has been shown that cytoplasmically inherited oligomycin resistant mutants exhibit various levels of resistance to oligomycin at the level of sub mitochondrial particles and that these mutants map at three different loci on the mitochondrial genome (Avner and Griffiths, 1970; 1973).

Reconstitution experiments have been performed using $F_1$ ATPase and OSCP from a common source and $F_1$ ATPase , OSCP depleted membranes from mutant and wild type strains. The cytoplasmically inherited factor(s) responsible for altered

TABLE 2.4

Binding of $^{3}H$ -NEM to Wild Type and Mutant Mitochondrial Membranes

| $^{3}H$ | nanomoles of $^{3}H$ NEM bound per mg protein | | | |
|---|---|---|---|---|
| nmoles NEM added per mg protein | D22 | A21 | A19 | A15 |
| 1 | 0.63 | 0.68 | 0.75 | 0.65 |
| 5 | 2.27 | 2.0 | 2.12 | 2.31 |
| 10 | 2.5 | 2.57 | 2.58 | 2.44 |
| 20 | 3.49 | 3.24 | 3.37 | 3.68 |
| 40 | 4.5 | 4.34 | 4.0 | 4.32 |

oligomycin sensitivity in all cases are localised within the $F_1$ ATPase, OSCP depleted membranes and are probably represented in the four mitochondrially synthesised peptides which make up the membrane fraction of the oligomycin sensitive ATPase (Tzagoloff, 1973). Similar results have recently been published by Shannon et al. (1973) in studies on cytoplasmically inherited oligomycin resistant mutants.

The reconstitution experiments described in this chapter employ hybrid enzyme reconstituted from diploid bakers yeast $F_1$ ATPase and OSCP and haploid D22 and mutant depleted membranes. Whilst the inclusion of a wild type enzyme in an experiment involving several mutants constitutes in itself a very powerful control, a more ideal situation would have included reconstitution experiments between wild type membranes and mutant $F_1$ ATPase and OSCP and vice versa. This type of control was attempted but the time needed to prepare amounts of wild type and mutant $F_1$ ATPase and OSCP required to carry out a comprehensive control would not have been justified by the experiments limited place in this thesis. Table 2.3 shows that there were no gross differences in specific activities, between wild type and mutant, in either the SMP or the reconstituted membranes. This would indicate a similar reconstitution mechanism in all strains with no mutant reconstituting an abnormally high or low membrane bound activity. Column 1 of Table 2.1 indicates that very little non specific binding of $F_1$ ATPase is taking place and so the decreased final levels of inhibition seen in the reconstituted enzyme would probably not be due to small amounts of insensitive $F_1$ being pelleted with the reconstituted membrane. A further indication that no

non specific binding is taking place is that a very small quantity of insensitive $F_1$ ATPase, with an expected specific activity between 40 and 60 would dramatically boost the specific activity of any contaminated reconstituted membrane and this insensitive activity would diminish to negligible proportions any sensitive reconstituted enzyme with a specific activity of approximately 1. It is felt, therefore, that these results do represent a true indication of altered membrane factors affecting enzyme sensitivity and that the differentials in sensitivity, between mutant and wild type, shown in SMP are broadly maintained in the reconstituted enzyme.

The gross mitochondrial membrane structure of wild type and mutant cells have been studied using fatty acid spin labels and $^{3}$H-NEM as structural probes. In the case of all the mutants tested no large discrepancy in membrane structure by the criterion of E.S.R. or NEM labelling was apparent. In conclusion we may say that the factor(s) responsible for the lowered sensitivity of the mutants to oligomycin appear to reside in the membrane fraction of the OS ATPase. The manifestation of the mutation does not, however, appear to cause any large disruption of the membrane structure and bring about the resistance phenomena in this way but is probably a much more subtle change probably in the OS ATPase complex and affecting an inhibitor binding site.

# CHAPTER 3

## PREPARATION AND SOME PROPERTIES OF AN INHIBITOR SENSITIVE, SOLUBLE MITOCHONDRIAL ATPase

### 3.1 INTRODUCTION

In this chapter the preparation and some relevant properties of an inhibitor sensitive, soluble mitochondrial ATPase preparation are presented. At least three laboratories have isolated and defined mutations in the mitochondrial DNA which confer resistance to oligomycin on the mitochondrial ATPase complex of yeast both in *vivo* and in *vitro* (Stuart, 1970; Avner and Griffiths, 1970; Criddle *et al*. 1973). However the in vitro inhibitor studies have either been carried out on mitochondria, sub mitochondrial particles or particles reconstituted from the three major components of the ATPase complex, that is 'stripped' membrane, oligomycin sensitivity conferring protein and $F_1$ ATPase, from parental and mutant cells. The results of such reconstitution experiments indicate that the resistance factor resides in the membrane fraction of the ATPase complex. However oligomycin sensitive ATPase activity also depends on membrane lipids (Bulos and Racker, 1968). Indeed Swanljung *et al*., (1972) reported a lower content of associated ergosterol as characteristic of ATPase purified from two class ll mutants although a reinvestigation of these mutants and their parental strain shows no significant differences.

From these studies one can reasonably draw the conclusion that the resistance factor(s) lie in the membrane, but whether the lesion in the mDNA leads to an altered inhibitor binding site located on the hydrophobic membrane peptides of the ATPase

complex, or whether the lesion is regulatory and leads to an altered lipid composition or arrangement of the membrane thus altering the sensitivity, or whether the lesion is responsible for a conformational change and thus denies the inhibitor access to its binding site still remains unclear.

A different approach presented in this chapter is to study the effect of various inhibitors on a membrane free, non particulate enzyme. A study of such an enzyme functioning free from any membrane directed constraints and in a grossly modified lipid environment, amenable to manipulation and yet still displaying the inhibitor resistances and/or sensitivities shown at the mitochondrial level would lend strong support to the supposition that the lesion in the mDNA is leading to an altered mitochondrially synthesized peptide residing in the membrane fraction of the ATPase complex and that the resistance phenomena is due to an altered inhibitor binding site.

Various oligomycin sensitive ATPase preparations have been reviewed in the general introduction. For this study the preparation of Tzagoloff and Meagher (1971) was chosen because while being a simpler preparation gave both a better recovery of ATPase activity, some 22% of SMP activity being recovered in the pure enzyme, and a higher specific activity, rising from 2.3 μmole Pi/min/mg in the SMP to 28.0 μmole Pi/min/mg in the purified enzyme, than the possible alternative preparation of Swanljung (1973).

For these reasons a slightly modified version of the Tzagoloff preparation was chosen for these studies.

The genetic studies of Lancashire and Griffiths (1975) indicated interaction between different binding sites on the inner mitochondrial membrane of yeast for the inhibitors TET and

oligomycin. In view of the importance of this in identifying physically proximal sites of inhibition of these two inhibitors it was decided to investigate this phenomenon at the level of sub mitochondrial particles and solubilized enzyme. As can be seen from Table 3.1 the insertion of $OLY^R$ phenotype into mitochondrial triethyl tin resistant mutants markedly lowers the resistance to TET. It is not possible to see if a converse interaction is taking place in whole cell studies because levels of oligomycin which can be used on plates are insufficient to prevent growth of cells mutated at locus OL1, however at the mitochondrial and soluble enzyme level sufficient levels of inhibitor can be used and a further investigation of any interaction is therefore possible.

## 3.2 METHODS AND MATERIALS

### Yeast Strains

The haploid strain used routinely in this investigation for isolation of the mutants was:-
D22 a $ad_2^-$ $arg^+$ $met^+$ $\rho^+$ $\omega^+$ $C^S$ $Er^S$ $OL^S$ $Sp^S$ $Cy^S$ $CP^S$. This strain was the gift of Dr. D. Wilkie. The mutants were produced by ultraviolet irradiation and selected as described by Avner and Griffiths (1970; 1973).

The constructed diploid strains used in this investigation were derived as shown in Table 3.2. D22/EC6 being a cytoplasmically inherited mutant selected as resistant to triethyl tin (Lancashire, 1974). DP1-1B7$^e$ is an oligomycin resistant mutant selected in the laboratory of Professor P.P. Slonimski and mapping at locus OL1.

### Materials

TABLE 3.1

In Vitro Resistance of Constructed Diploids to Triethyl Tin

| Strain | Genotype | Resistance to TET (μM) |
|---|---|---|
| KL110 | $O^S T_{16}{}^R$ | 40 |
| KL111 | $O_7{}^R T_{16}{}^R$ | 10 |
| KL113 | $O_7{}^R T^S$ | ? |
| KL114 | $O^S T^S$ | ? |

Phenotypic Interaction between Oligomycin Resistance and TET Resistance in Isonuclear Constructed Diploids.

Diploid strains were assayed for tolerance to TET using the Drop out procedure on glycerol medium containing the Following concentrations of TET: 2, 4, 10, 20, 40 and 80 μM. Growth was scored after three days incubation at 30°C.

See Table 3.2 for origin of strains. The data for this table was provided by Dr. W. E. Lancashire.

TABLE 3.2

Genetic Derivation of Constructed Diploids

| Strain | Genotype Constructed Diploid Strains | Origin of Strain |
|---|---|---|
| KL110 | a ade + + + his trp + $O^{S}T_{16}{}^{R}$ | D22/EC6 x DP1-1B/7$^{e}$ |
| KL111 | a ade + + + his trp + $O_{7}{}^{R}T_{16}{}^{R}$ | D22/EC6 x DP1-1B/7$^{e}$ |
| KL113 | a ade + + + his trp + $O_{7}{}^{R}T^{S}$ | D22/EC6 x DP1-1B/7$^{e}$ |
| KL114 | a ade + + + his trp + $O^{S}T^{S}$ | D22/EC6 x DP1-1B/7$^{e}$ |

The data provided in this Table was from Dr. W. E. Lancashire.

Venturicidin was a gift from Dr. I.D. Fleming, Glaxo Laboratories, Stoke Poges, U.K. and can now be obtained from British Drug Houses Ltd., Poole, Dorset, U.K.

Oligomycin was obtained from Sigma Chemicals Inc.

Growth of Saccharomyces cerevisiae

Cells were grown and harvested as described in Chapter 2.

Preparation of Asolectin

Purified soya bean asolectin was prepared as described in Chapter 2.

Preparation of SMP

SMP were produced as described in Methods and Materials, Chapter 2.

Protein Estimations

Protein estimations were described in Methods and Materials, Chapter 2.

Preparation of Triton Extracts

To a suspension of yeast sub mitochondrial particles in 0.25 M Tris acetate pH 7.5 at 25 mg protein/ml was added 2.9 volumes of 5 mM Tris acetate pH 7.5. The solution was mixed well and a 10% w/v solution of Triton X-100, in 5 mM Tris acetate pH 7.5, was added slowly with stirring to give a final Triton X-100 concentration of 0.25%. The solution was centrifuged at 100,000 g for 20 min. The lipid layer and pellet were discarded, only the clear infranatant being retained.

Purification of Inhibitor Sensitive ATPase

50 mls of 5 to 20% linear sucrose gradients were prepared

containing 0.05% Triton X-100, 10 mM Tris acetate pH 7.5 using a simple two chambered device. Stirring was effected by a Teflon coated spiral of wire driven by a variable speed overhead stirrer. The gradients were built in Beckman SW 25.2 tubes and chilled at 4°C for several hours before use. 5 ml of Triton X-100 extract was carefully applied per gradient and the gradients spun at 23,000 r.p.m. for 20 hours in a Beckman L2-50 ultracentrifuge which was stopped without the brake. Ten 5.5 ml fractions were collected from the bottom of the tube by pumping through a multi-channel variable speed peristaltic pump.

100 µl of each fraction was assayed for ATPase activity in the presence of 50 µl of purified asolectin. The active fractions were pooled and either used directly or concentrated as indicated. The enzyme was frozen in a mixture of cardice and acetone and stored at -30°C. The procedure is summarized in Fig. 3.1.

Assay of ATPase Activity

ATPase activity was measured in a reaction mixture containing 5 mM ATP, 2 mM $MgCl_2$, 50 mM Tris-HCl, pH 7.5 to 10.0 or 50 mM Tris maleate pH 6.0 to 7.5 in a final volume of 1 ml. In assays on crude Triton X-100 extracts 60 to 80 µgms of protein were added and in assays on purified enzyme 15 to 25 µgms of protein were added. All inhibitors were added as concentrated ethanolic solutions, the ethanol never exceeding 1% in the final assay mix. In a control experiment addition of ethanol alone had no effect on ATPase activity over the time span of the assays (Figure 3.0M). Enzyme and inhibitor were preincubated together for 5 minutes at 30°C prior to initiation of the reaction with ATP. The reaction was allowed to proceed for five or ten

FIGURE 3.1

Flow Chart

Sub mitochondrial particles in sucrose tris (25 mg/ml).

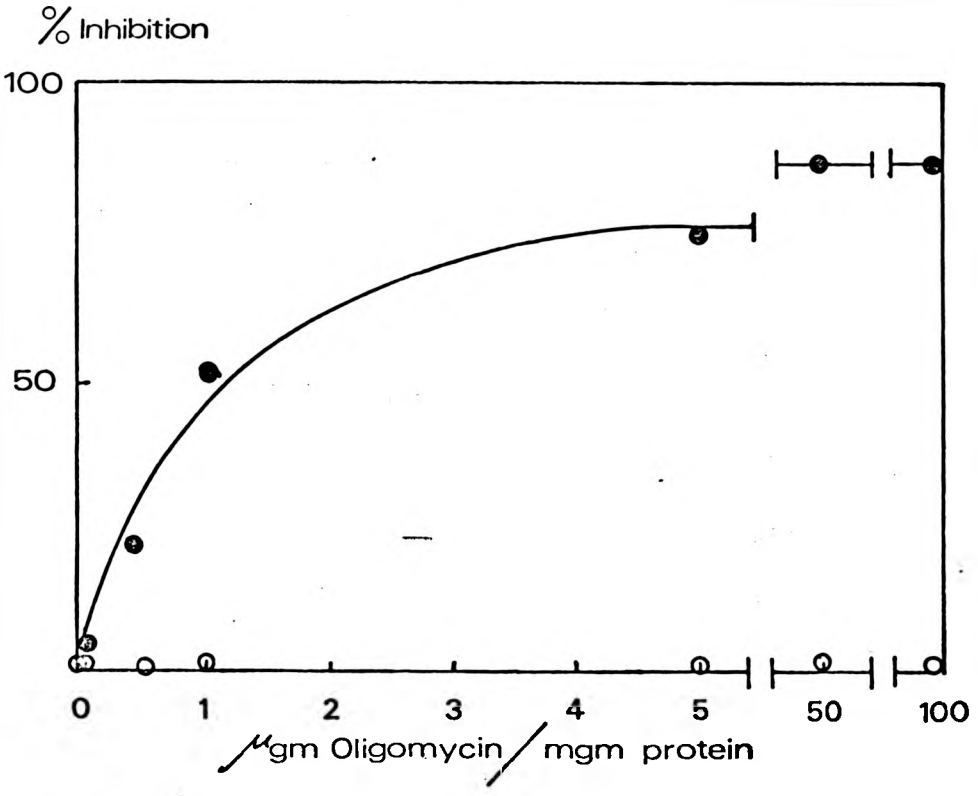
↓

Add 2.9 vols 10 mM Tris acetate pH 7.5 and 0.1 vol. 10% w/v Triton X-100 slowly with stirring. Spin 105,000 g x 20 mins.

↓ → Pellet and lipid discarded.

Infranatant removed with Pasteur pipette. 5 ml layered onto 50 ml 5 - 20% linear sucrose gradient containing 10 mM Tris acetate pH 7.5, 0.05% Triton X-100. Spun 18 - 20 hours in a Beckman SW25.2 head, 23,000 r.p.m. Gradient fractionated into 11 equal fractions. 100 µl of each fraction was assayed for ATPase activity in the presence of 550 µgms purified asolectin. The most active fractions were pooled. All steps were carried out at 0 - 4°C.

FIGURE 3.1m




ATPase Assay: effect of ethanol

16.6 µgm of gradient purified D22 OS ATPase was assayed in in the presence of ethanolic solution of inhibitor (●——●) and in the presence of equivalent amounts of ethanol (o——o) over the same incubation times.

minutes as indicated and was stopped by the addition of 1 ml 10% (w/v) trichloracetic acid. The reaction was always linear over the time course followed. The mixture was centrifuged at 6,000 r.p.m. for five minutes to sediment the protein. 0.5 ml samples of the supernatant were removed for the determination of phosphate (King, 1932).

Preparation of Samples for Gel Electrophoresis

To 800 µgms protein in 2 ml 10 mM Tris acetate pH 7.5 was added 8 ml acetone and 0.1 ml 2M KCl, the solutions were thoroughly mixed and left overnight at 0°C in conical glass test tubes. The precipitated protein was spun down at 3,000 r.p.m. for 10 minutes and the supernatant decanted off, excess acetone was dried off in a gentle air stream. The pellet was resuspended in 0.8 ml of a mixture containing 1% w/v SDS, 10% w/v glycerol, 10 mM $Na_2HPO_4$ and 1% v/v mercaptoethanol and thoroughly broken up with a glass rod. The tubes were capped with aluminium foil and placed in a boiling water bath for one minute and then transferred to a thermostatted water bath at 70°C for a further 20 minutes. 10 µl of bromophenol blue was added as a tracking dye and the appropriate volume of the mixture (final protein concentration 1 mg/ml) applied to the top of the gel.

Gel Electrophoresis in SDS

The conditions of gel electrophoresis were essentially those of Weber and Osborn (1969). The final concentration of acrylamide was 7.5% and the ratio of monomer to cross-links was 36.8 : 1 w/w. Gel dimensions were 75 mm by 5 mm. Electrophoresis was halted when the bromophenol blue tracker dye was within 15 mm of the end of the gel.

## 3.3. RESULTS

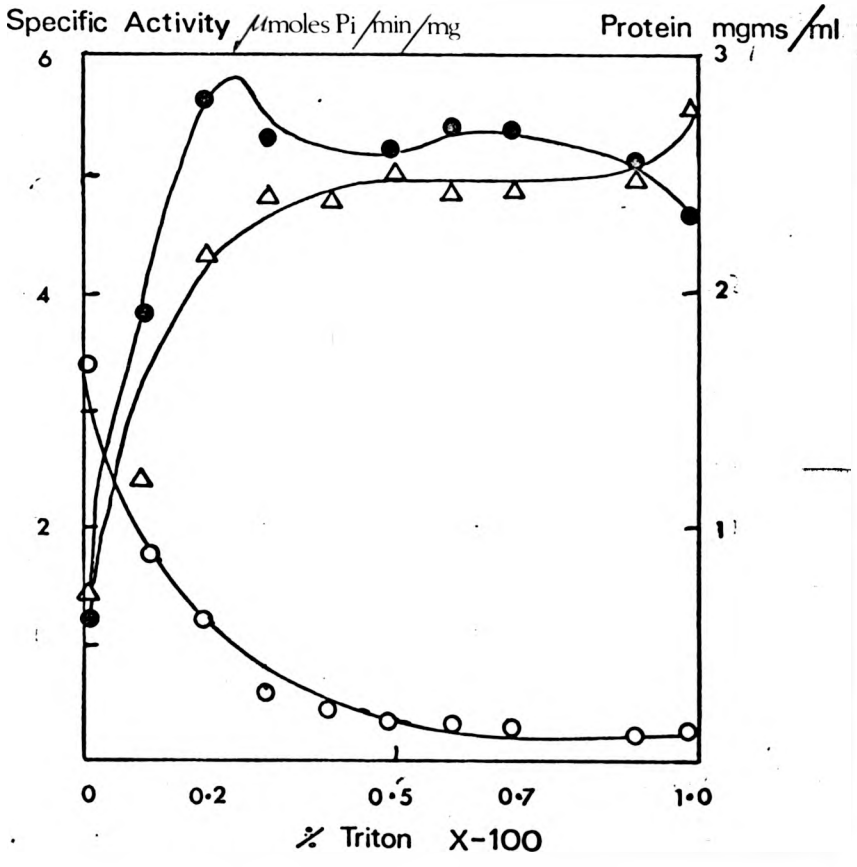
### Preparation of Inhibitor Sensitive ATPase

The removal of ATPase from sub mitochondrial particles by various concentrations of Triton X-100 is demonstrated in Fig. 3.2. It can be seen that the bulk of the ATPase activity is removed with a Triton concentration of between 0.2 and 0.3% w/v and that subsequent increases in the Triton concentration have little additional effect on removal of either ATPase activity or other protein species. It was therefore decided to use a Triton concentration of 0.25% in subsequent enzyme preparations to maximise enzyme recovery while keeping the Triton concentration as low as possible for reasons given later.

The recovery of enzyme throughout the procedure is given in Table 3.3. A typical gradient profile is given in Fig. 3.3 from which it can be seen that the bulk of the protein in the crude Triton extract consists of low molecular weight contaminants. The difficulties encountered by Broughall (1973) in using this procedure on yeast strain D22, such as cold lability of wild type enzyme sensitivity and low final specific activity of enzyme were not encountered. Indeed no significant change in wild type enzyme sensitivity to oligomycin was detectable after the enzyme had been removed from the membrane for 18.75 hours (see Fig. 3.4). This contrasts remarkably with the findings of Broughall who found the wild type enzyme sensitivity to oligomycin to be very unstable, losing meaningful sensitivity 270 minutes after removal from the membrane and to be completely insensitive to oligomycin 24 hours after removal from the membrane. In contrast to this he reported that the soluble ATPase from the mutant strains he investigated to have a stable sensitivity over

FIGURE 3.2

The Solubilizing Effect of Triton X-100 on Yeast SMP ATPase



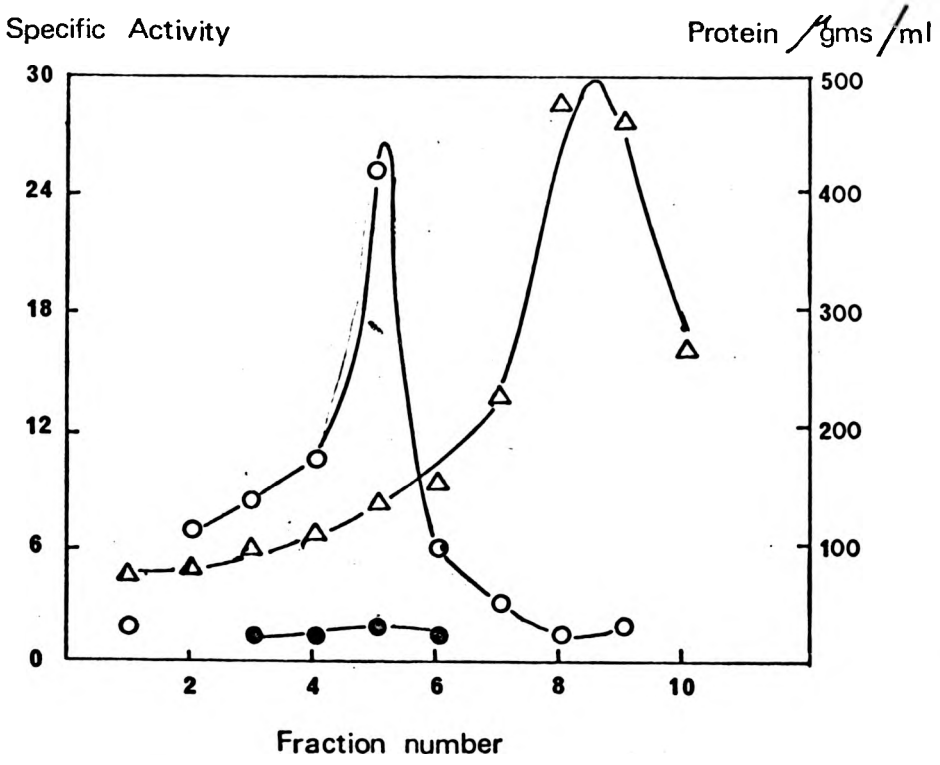

SMP at a protein concentration of 25 mg/ml were diluted, with the appropriate volumes of 10 mM Tris acetate, pH 7.5 and 10% w/v Triton X-100 to give a final protein concentration of 6.25 mg/ml and the Triton concentrations indicated. The solutions were centrifuged at 105,000 g for 20 min and the pellet (o——o) and supernatant (●——●) assayed for ATPase activity, and the protein concentration (mg/ml) determined (△—△).

TABLE 3.3

Recovery of Protein and Enzyme Activity throughout OS ATPase Purification

| Fraction | Protein mg | Specific Activity | Specific Activity + 5/$\gamma$g Ven/ml | Total Units ATPase |
|---|---|---|---|---|
| Mitochondria | 941 | 6.1 (3.6 - 6.1) | 0.52 | 5672 |
| S.M.P. | 603 | 6.06 (6.0 - 6.6) | 0.55 | 3654 |
| Triton Extract Infranatant | 193 | 10.7 (7.0 - 11.2) | 1.11 | 2098 |
| Triton Extracted Pellet | 248 | 2.3 | 0.63 | 571 |
| Gradient Purified Enzyme | 45.5 | 29.82 | 2.18 | 1519 |

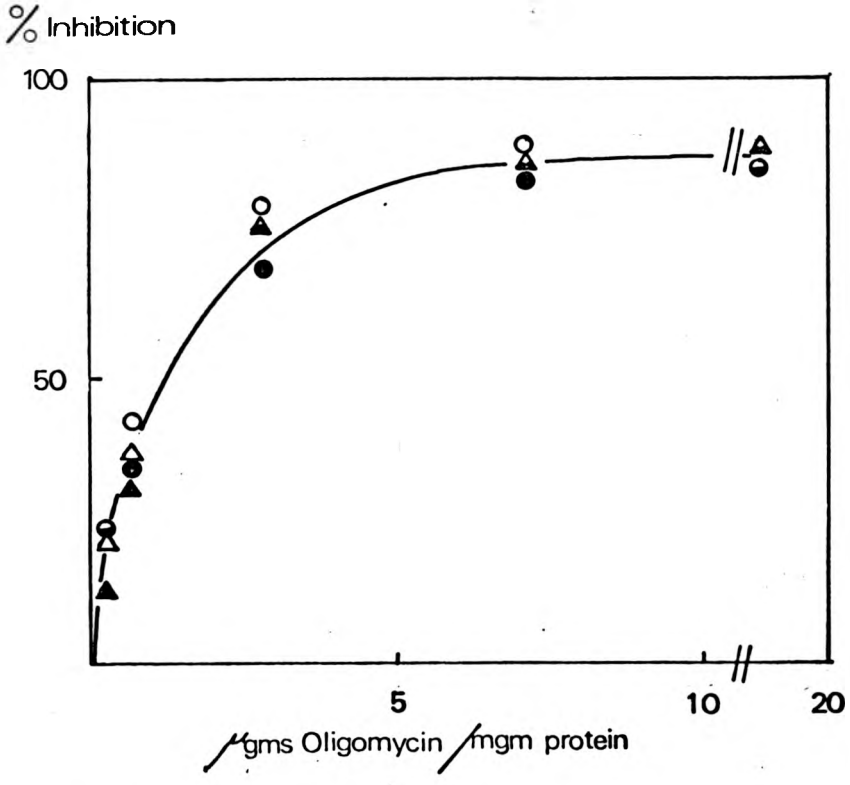
.FIGURE 3.3




The Gradient Profile Obtained during OS ATPase Purification

A gradient profile of a 50 ml, 5 - 20% linear sucrose gradient containing 10 mM Tris acetate, pH 7.5 and 0.05% Triton X-100. 5 ml of Triton extract infranatant was loaded on top of the gradient which was spun for 20 hours in a Beckman SW 25.2 rotor. 11 fractions of 5.5 ml were collected and ATPase activity was assayed as outlined in Fig. 3.1, in the absence (o——o) and presence (•——•, 5 /µg/ml) of venturicidin. Specific activities are expressed in /µmole Pi/min/mg protein. Protein concentrations are expressed in /µg/ml (Δ——Δ). Fraction 1 is the bottom fraction of the gradient.

FIGURE 3.4




Cold Stability of OS ATPase

D22 SMP at 25 mg/ml were diluted with 2.9 volumes of ice cold Tris acetate pH 7.5 and 0.1 volume of 10% w/v Triton X-100 was added slowly with stirring. The suspension was centrifuged at 105,000 g for 20 min. The infranatant (30 μl per ml assay volume) was assayed for inhibitor sensitivity 75 min (Δ—Δ), 165 min (o—o), 375 min (●—●) and 18.75 hours (▲—▲) after removal from the membrane. All operations were carried out at 4°C.

these time spans. In this study the parental and mutant strains all displayed stable responses to the various inhibitors tested and as can be seen from Fig. 3.5, a typical inhibition curve for an enzyme preparation frozen in cardice-acetone mixture, these sensitivities survive at least one freeze-thaw cycle. Broughall reported a decrease in wild type enzyme specific activity during purification, i.e. from 0.72 μmoles Pi/min/mg in sub mitochondrial particles to 0.63 μmoles Pi/min/mg in the purified enzyme, this was not found in this study.

The Effect of Triton X-100 Concentration on Enzyme Activity and Oligomycin Sensitivity

Table 3.4 shows the inhibitory effect of Triton X-100 on purified enzyme. In view of this finding the Triton concentration was kept to a minimum in subsequent assays.

The critical micelle concentration of Triton X-100 is reported to be between 0.01 and 0.017% w/v depending on the buffer system used (Rohm and Haas, 1969) and during attempted removals of the detergent from solubilised membranes it has been reported to behave as a large molecular weight aggregate of approximately 100,000 Daltons. Assuming a micelle molecular weight of these proportions attempts to concentrate the enzyme by ultrafiltration through Diaflow XM50 or XM100 membranes (Tzagoloff, 1971; Broughall, 1973) or through Minicon B. macrosolute chambers or centriflo cones with a molecular weight cut off of about 5,000 Daltons (Cosson and Spiridakis, 1974) would result in a rapid concentration of Triton X-100 in the enzyme sample. This increase in detergent levels in the assay system would grossly modify the apparent response of the enzyme to lipophillic inhibitors (Tzagoloff, 1971). Fig. 3.6 shows that

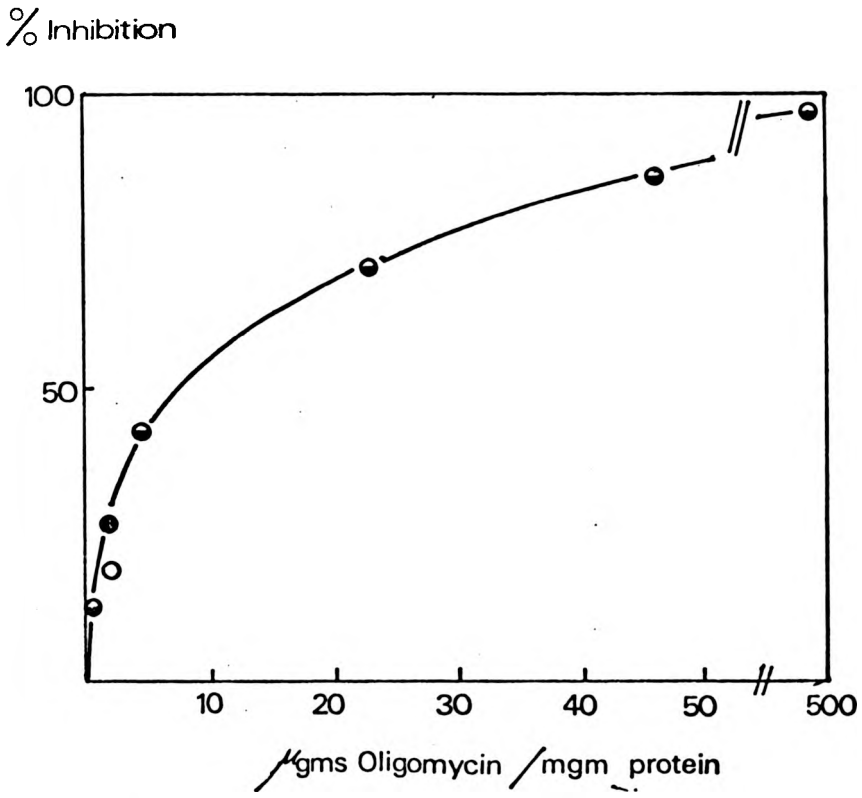
TABLE 3.4

The Effect of Triton X-100 on the Specific Activity of OS ATPase

| Assay Concentration (% w/v) of Triton X-100 | Specific Activity μmoles/min/mg protein |
|---|---|
| 0.005 | 13.7 |
| 0.01 | 9.1 |
| 0.02 | 6.2 |

Concentrated OS ATPase was assayed in the absence of exogenous phospholipids in the presence of the indicated concentration of Triton X-100.

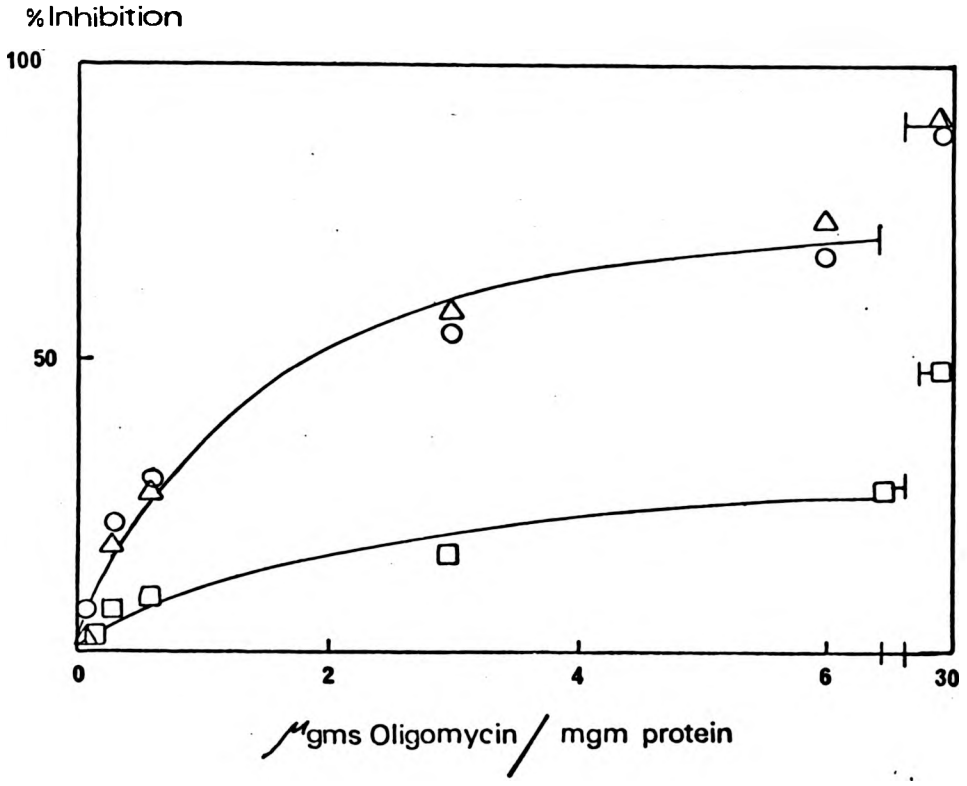
FIGURE 3.5




The Stability of OS ATPase to Freezing and Thawing

Purified D22 OS ATPase was concentrated to 2.15 mg protein per ml in a Minicon B concentrator (Amicon Corp., Lexington, Mass., U.S.A.) and assayed for ATPase activity in the absence of added phospholipid before (o——o) and after (●——●) freezing in a mixture of cardice and acetone.

FIGURE 3.6




The Effect of Triton X-100 Concentration on Enzyme Sensitivity

D22 unconcentrated OS ATPase was assayed in the absence of exogenous phospholipid and in the presence of 0.005% (o——o), 0.01% ( Δ—Δ ) and 0.02% (□—□ ) Triton X-100.

even a doubling of Triton X-100 in the assay system dramatically alters the inhibitory effect of oligomycin on the wild type enzyme. It may well be significant that various levels of Triton below the critical micelle concentration do not seem to have an adverse effect on enzyme sensitivity but once the detergent is allowed to form micelles, i.e. at 0.02% w/v, the apparent sensitivity of the enzyme to inhibitor is greatly reduced. The concentration of Triton in the assay medium, because of the dual effect of enzyme inhibition and inhibitor sequestration, is therefore of critical importance in comparitive studies of enzyme activities and the actions of lipophillic inhibitors.

In order to reduce the Triton concentration, in the assay system, to a minimum the following steps were taken:-

1) The concentration of Triton in the gradients was reduced to 0.05% w/v. Attempts to reduce this still further i.e. to 0.01% resulted in a complete loss of enzyme activity in the gradient.

11) The enzyme recovered from the gradient was not concentrated but added directly to a final volume for the assay of 1 ml, usually 100 µl was added containing 15 - 20 µg protein, resulting in a final Triton concentration of approximately 0.005% w/v, considerably lower than the critical micelle concentration.

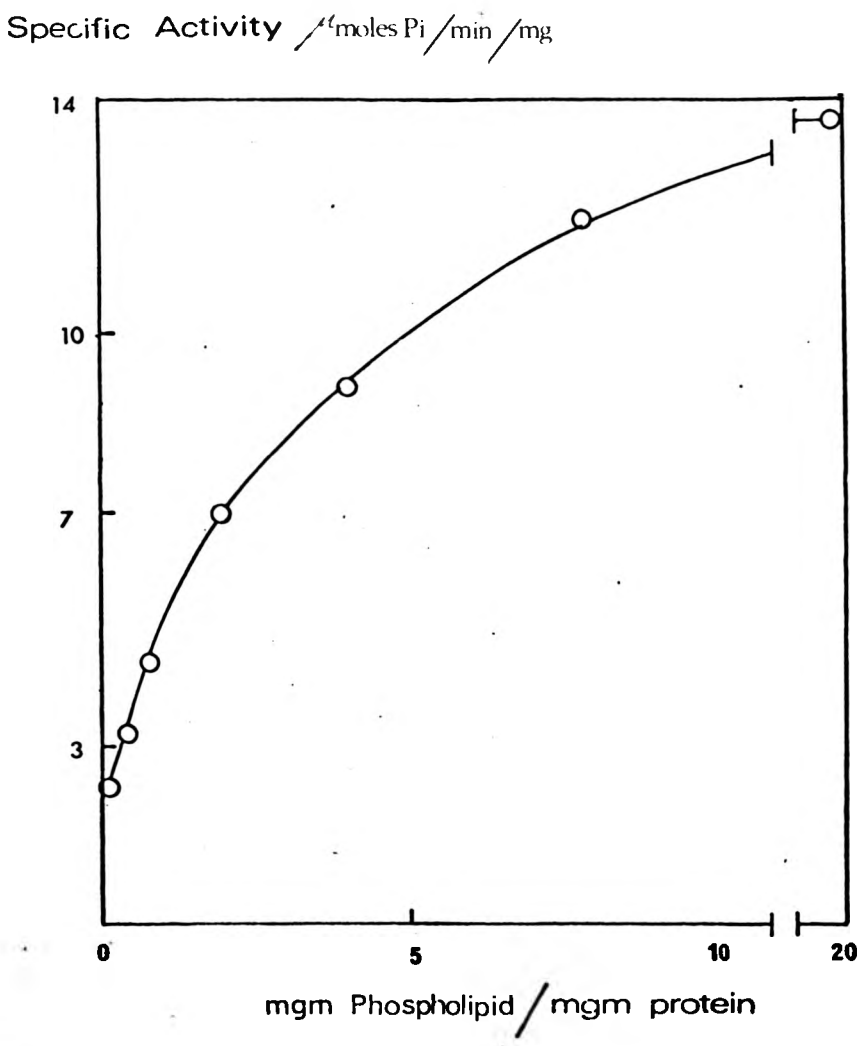
Effect of Phospholipids on Enzyme Activity

The ATPase activity recovered from the gradient does not require exogenous phospholipid to be manifest (Tzagoloff, 1971) but as shown in Fig. 3.7 can be markedly stimulated by the addition of phospholipid.

Effect of Phospholipids on Enzyme Sensitivity

The addition of phospholipids to the assay medium drastically

FIGURE 3.7




The Effect of Phospholipids on Enzyme Activity

D22 OS ATPase was concentrated approximately 10 fold to a protein concentration of 2.16 mg/ml and assayed for activity with the indicated amounts of phospholipids.

alters the sensitivity of the enzyme to the lipophillic inhibitors oligomycin, venturicidin and triethyl tin (see Figs. 3.8, 3.9 and 3.10). This effect is probably analogous to the effect of increasing Triton concentrations and is due to the additional lipid competing with the enzyme for inhibitor and resulting in an apparent resistance. This difficulty was overcome by assaying only the residual largely lipid depleted activity recovered from the gradient and extending the assay incubation period to compensate for the lower specific activity rather than increasing the amount of enzyme which would have resulted in an increased Triton concentration.

Disc Gel Electrophoresis

No gross differences in the sedimentation characteristics of the purified enzyme from mutants A21, A19, A15 and D22 parental cells were discernible, indeed no differences were discernible in several triethyl tin and venturicidin resistant mutants also investigated. This lack of any gross discernible change in enzyme integrity and molecular weight is reflected in the peptide patterns obtained with polyacrylamide disc gels (Fig. 3.11). The sedimentation characteristics and gel patterns would seem to indicate that the mutation leading to the resistance phenomenon is not a gross modification of the enzyme such as complete deletion of a peptide species and is of a much more restricted and less drastic nature.

Inhibitor Resistances in Crude Triton Extracts

An initial investigation into the sensitivities and/or resistances of inhibitor sensitive ATPases from parental and mutant strains was carried out on Triton extracts prior to gradient purification of the enzyme. The results of this

FIGURE 3.8

The Effect of Phospholipid on Oligomycin Sensitivity

D22 OS ATPase was concentrated approximately 10 fold to a protein concentration of 2.18 mg/ml and titrated against oligomycin in the presence of 0 (■—■), 0.717 (o—o), 1.42 (▲—▲), 3.58 (□—□), and 7.16 (Δ—Δ) mg of phospholipid/mg protein.

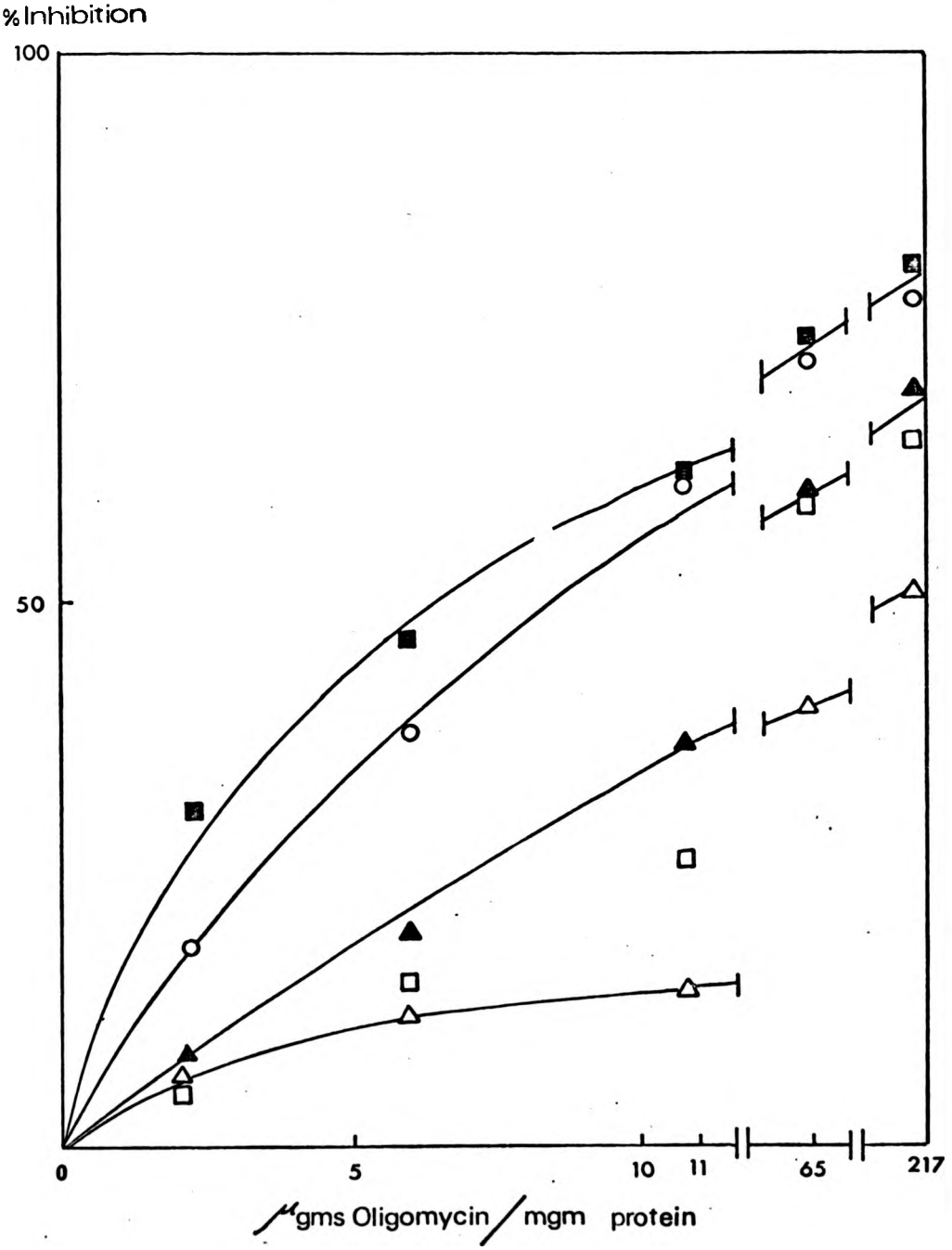

% Inhibition
100
50
0
5
10
11
65
217
μgms Oligomycin / mgm protein

FIGURE 3.9

The Effect of Phospholipid on Venturicidin Sensitivity

D22 OS ATPase was concentrated approximately 10 fold to a protein concentration of 1.8 mg/ml and titrated against venturicidin in the presence of 0 (■—■), 1.56 (o—o), 3.12 (▲—▲), 7.8 (□—□), 15.6 (△—△) and 112 (●—●) mg phospholipid/mg protein.

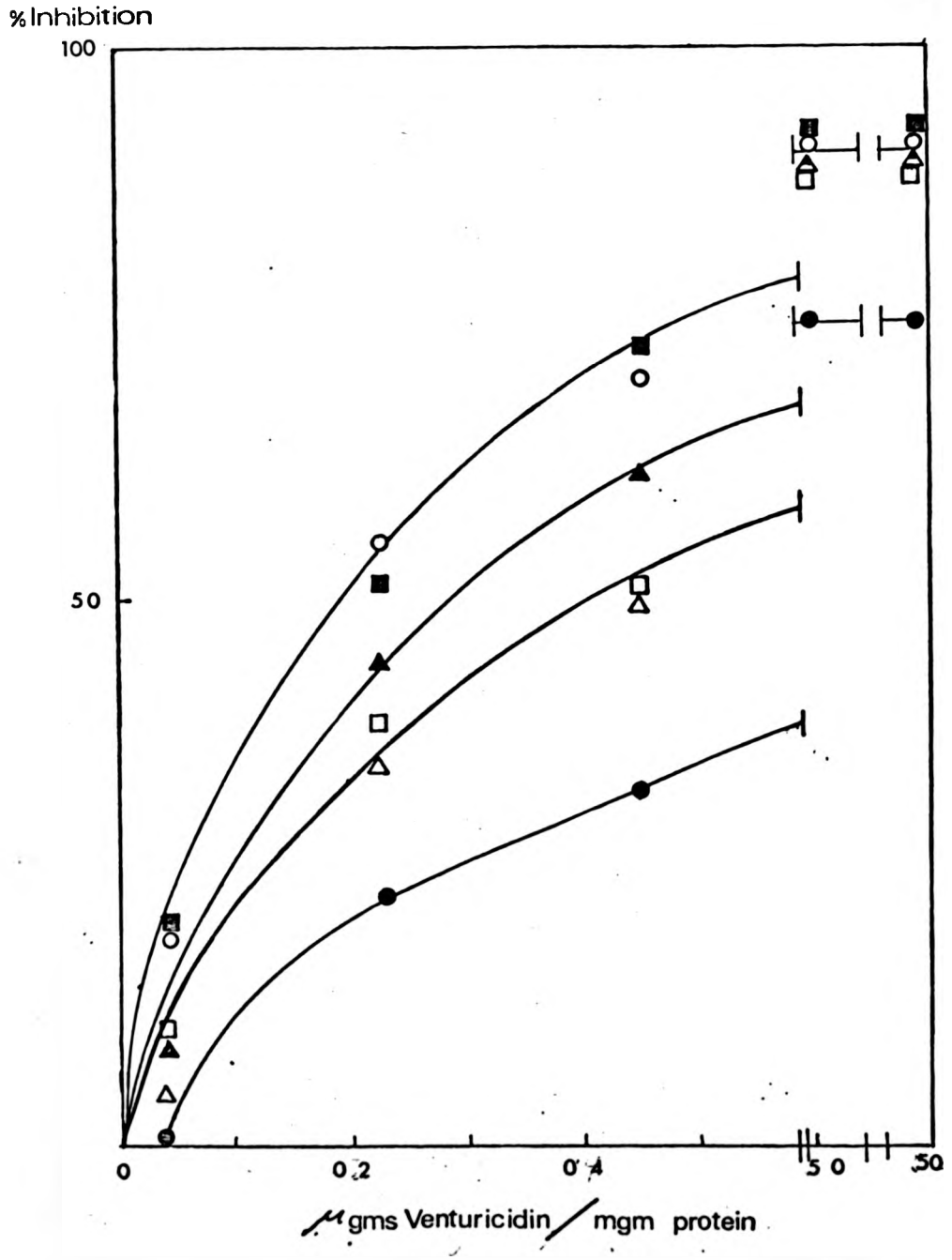

% Inhibition
100
50
0
0.2
0.4
5.0
50
μgms Venturicidin / mgm protein

FIGURE 3.10

The Effect of Phospholipids on TET Sensitivity

D22 OS ATPase was concentrated approximately 10 fold to a protein concentration of 1.8 mg/ml and titrated against TET in the presence of 0 (■—■), 1.56 (o—o), 3.12 (▲—▲), 7.2 (□—□), 15.6 (△—△) and 112 (●—●) mgs phospholipid/mg protein.

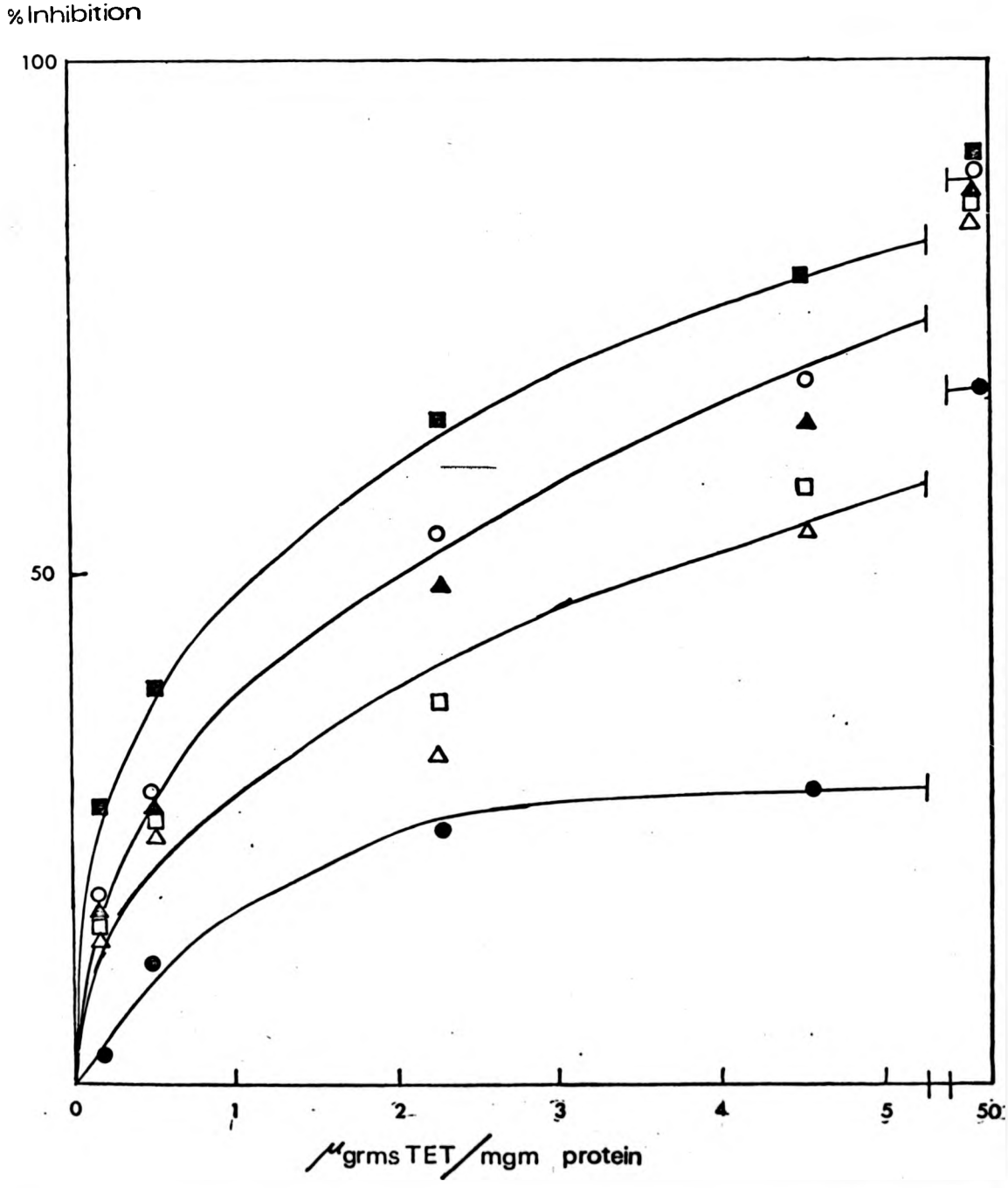
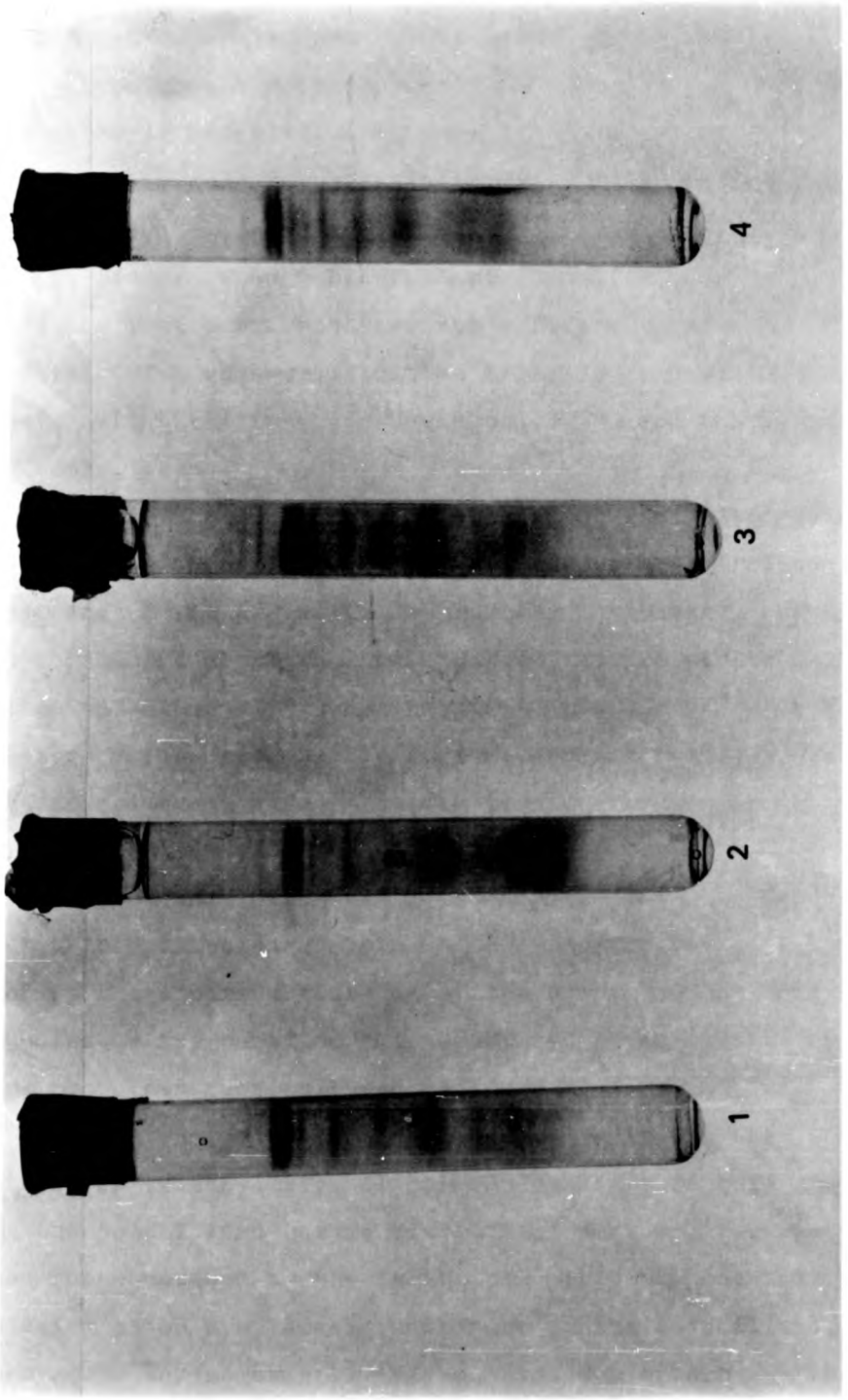

% Inhibition
100
50
0
1
2
3
4
5
50
μgrms TET / mgm protein

FIGURE 3.11

Oligomycin sensitive ATPase was prepared from strain D22 ( 1 ), A21 ( 2 ), A19 ( 3 ) and A15 ( 4 ). The enzyme was precipitated with acetone, solubilised and 100 $\mu$gm electrophoresed as described in Materials and Methods. The gels were stained and destained according to Weber and Osborn (1969).


4
3
2
1

investigation are summarised in Table 3.5.

Although the mutants used in this study were isolated on the criteria of their resistance to oligomycin the inhibitors triethyl tin and venturicidin were also used in the study of crude Triton extracts and purified inhibitor sensitive ATPase. As can be seen from Table 3.5 and Figs. 3.12, 3.13 and 3,14 both the Triton extracts and the purified enzymes exhibit sensitivities and specificities to inhibitors exhibited at whole cell (Griffiths and Houghton, 1974) and submitochondrial particle level.

Three factors expose the studies on the Triton extracts to the critiscism that they do not neccessarily reflect the reaction of the ATPase to the various inhibitors. These are:- 1) Fig. 3.3 shows that the bulk of the protein in the Triton extracts are in fact contaminants of the ATPase and these may in some way be responsible for the enzymes modified response to the inhibitors. 11) These extracts are heavily contaminated with lipids removed from the membrane by the Triton. 111) The extracts contain 0.25% w/v Triton and as seen in Fig. 3.6 even a ten fold dilution of this in the assay system will grossly modify the apparent response of the enzyme to the inhibitors. Studies on the purified enzyme and the assay conditions described are much less open to these criticisms.

The study of the purified enzyme indicates that the binding sites for triethyl tin, oligomycin and venturicidin are present in the solubilised enzyme complex and moreover the reaction of the three mutants to the inhibitors would suggest that the binding sites are largely unchanged in the solubilised, purified enzyme. Enzyme sensitivity to these inhibitors may therefore

TABLE 3.5

Cross Resistances in Crude Triton Extracts

| Strain | $I_{50}$ Oligomycin | TET | VEN |
|---|---|---|---|
| D22 | 3.0 | 0.36 | 0.11 |
| A15 | 9.7 | 0.28 | 0.12 |
| A21 | 19.0 | 0.32 | 0.18 |
| A19 | 50 | 2.9 | 0.50 |

0.25% Triton X-100 extracts were prepared as in Materials and Methods and their inhibitor sensitivity titrated. The $I_{50}$ value is the concentration in μgms/mg protein of inhibitor required to inhibit ATPase activity by 50%.

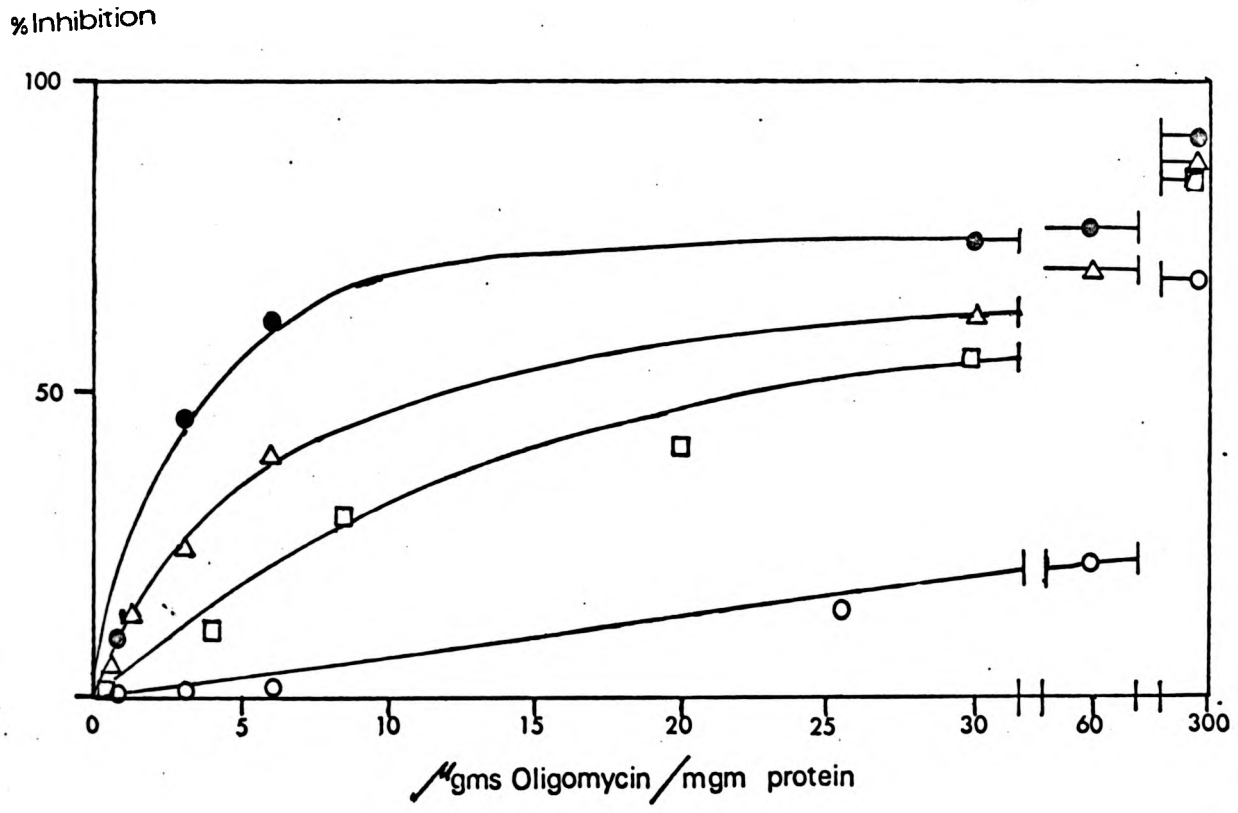
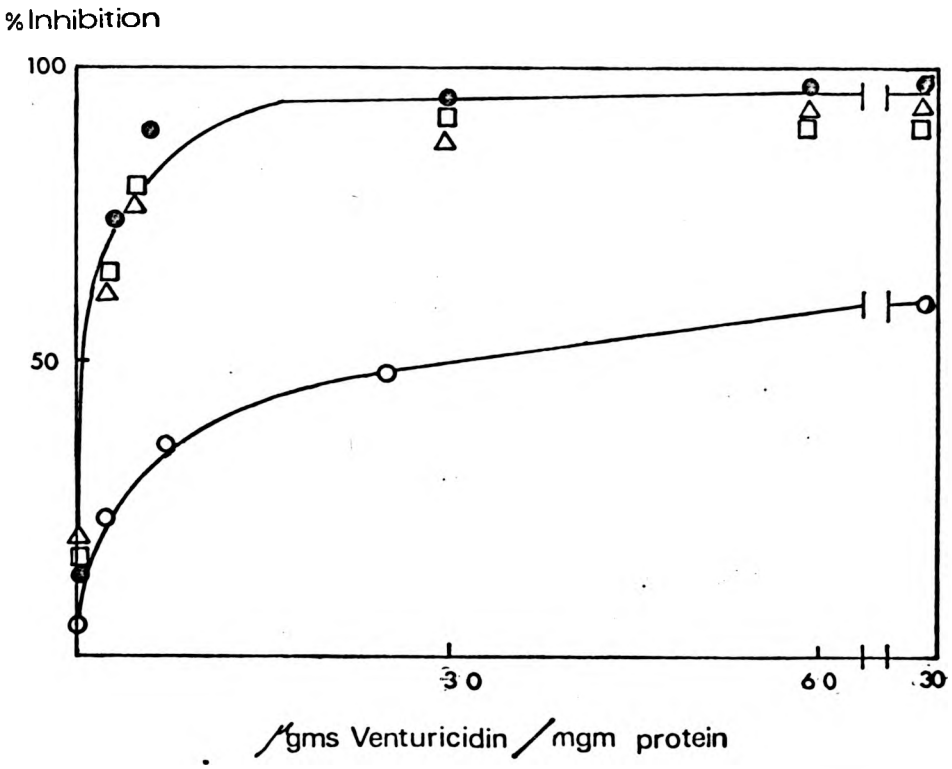



FIGURE 3.12 Oligomycin Sensitivity of OS ATPase from Wild Type and Mutants A21, A19 and A15.

OS ATPase from D22 (●—●), A21 (□—□), A15 (△—△) and A19 (○—○) was titrated unconcentrated against oligomycin. The conditions were as described in Materials and Methods. 15 - 20 μgms protein was used per assay.

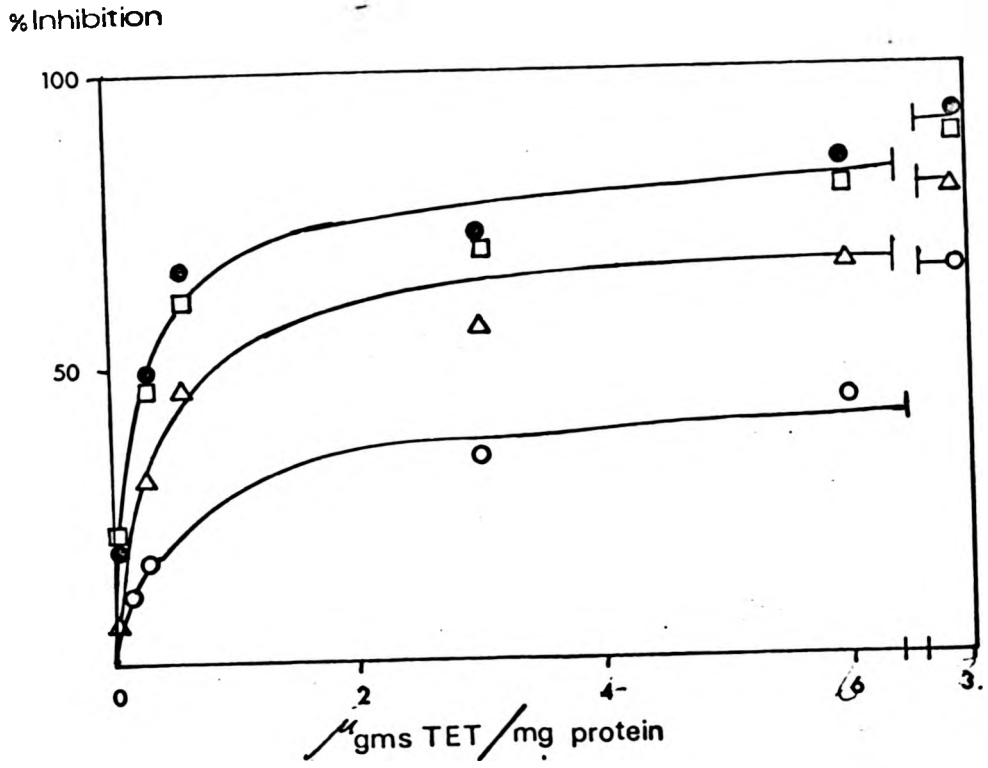
FIGURE 3.13




Venturicidin Sensitivity of OS ATPase from Wild Type and Mutants A21, A19 and A15.

OS ATPase from D22 (●—●), A21 (□—□), A15 (△—△) and A19 (o—o) was titrated, unconcentrated against venturicidin. The conditions were as described in Materials and Methods. 15 - 20 /gms of protein was used per assay.

FIGURE 3.14




TET Sensitivity of OS ATPase from Wild Type and Mutants A21, A19 and A15.

OS ATPase from D22 (●——●), A21 (Δ——Δ), A19 (o——o) and A15 (□——□) was titrated, unconcentrated against TET. The conditions were as described in Materials and Methods. 15 - 20 μgms of protein was used per assay.

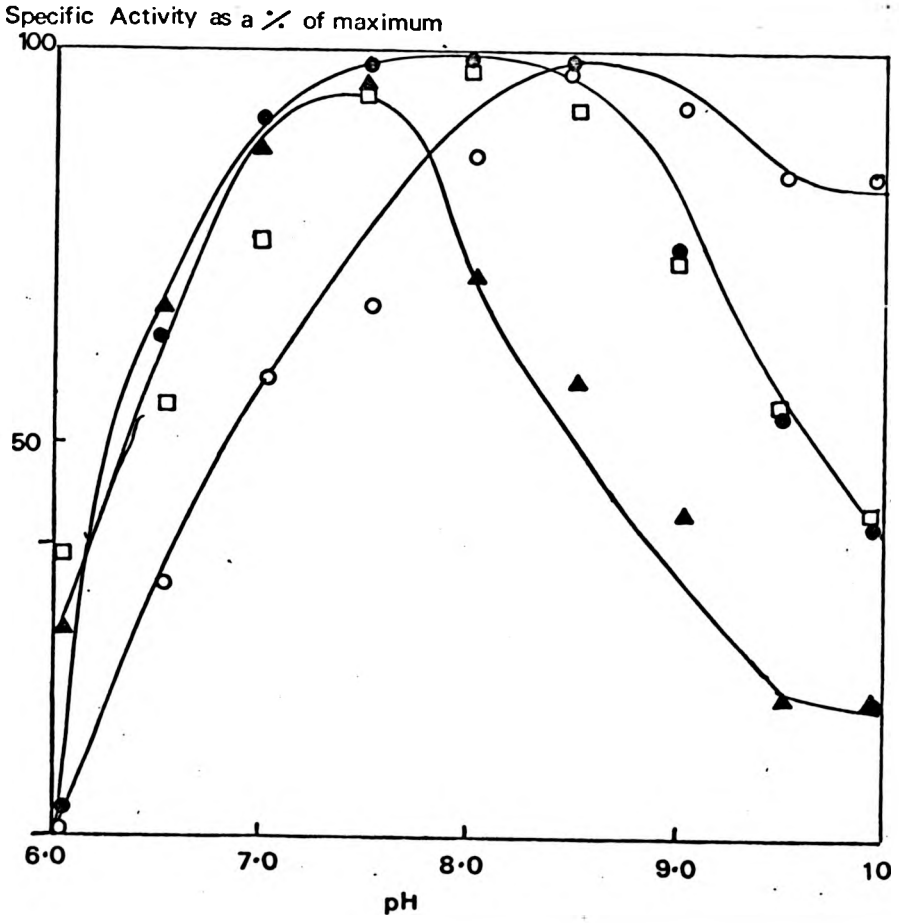
give an indication of the integrity of the enzyme preparation and their use gives the additional benefit of a control sensitivity or resistance.

The Effect of pH on Enzyme Activity and Oligomycin Sensitivity

Fig. 3.15 shows that the wild type enzyme has a broad pH optimum between pH 7.0 and 8.5. The pH profile from mutant A15 is very similar to that of the parental strain enzyme, while the shape of the profile is different from that recorded in mitochondria the agreement between these two strains correlates with the results found in SMP. The mutants A21 and A19 which display a much higher resistance to oligomycin than A15 and also map closely together, produce very different profiles both from the parental strain and from one another and display distinct and different pH optima. These results may be indicative of a mutational change of an ionising group in the purified enzyme.

Studies of the effect of changes of pH on oligomycin sensitivity of parental strain and mutants A21 and A19 have been carried out at the level of SMP (Griffiths and Houghton, 1974). The results presented here are a further investigation of these effects at the level of the purified enzyme. The differences in enzyme sensitivity of the wild type and mutant strains A21 and A15 found at the level of SMP were not apparent in the purified enzyme with the differentials in the oligomycin sensitivity of all the mutants investigated being maintained over a wide range of pH values as summarised in Table 3.6. Tris maleate and Tris acetate were found to be interchangeable at the lower pH values of 6.0, 6.5 and 7.0.

FIGURE 3.15




The Effect of Changes in pH on the Specific Activity of OS ATPas

Gradient purified, unconcentrated OS ATPase prepared from strains D22 (●—●), A21 (▲—▲), A19 (○—○) and A15 (□—□) was assayed at the various pH values indicated. At the pH values 6.0, 6.5 and 7.0 Tris maleate was substituted for Tris acetate.

TABLE 3.6

The Effect of Changes in pH on Inhibitor Sensitivity of OS ATPase

| pH | D22 | | | A21 | | | A19 | | | A15 | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | + oligo | - oligo | % I | + oligo | - oligo | %I | + oligo | - oligo | %I | + oligo | -oligo | %I |
| 6.5 | 2.41 | 3.92 | 73 | 5.77 | 7.8 | 26 | 6.99 | 8.19 | 15 | 5.06 | 8.92 | 43 |
| 8.0 | 2.77 | 12.29 | 77 | 8.81 | 11.9 | 26 | 14.94 | 16.63 | 10 | 8.44 | 16.39 | 49 |
| 9.5 | 0.48 | 6.03 | 92 | 3.32 | 4.2 | 21 | 15.4 | 16.63 | 7 | 5.3 | 8.92 | 41 |

Purified unconcentrated OS ATPase was assayed at the various pH values indicated in the absence of exogenous phospholipid and in the absence and presence (6 $\mu$gm/mg protein) of oligomycin. At the lower pH Tris maleate was substituted for Tris acetate.

Phenotypic Interaction Between Oligomycin Resistance and Triethyl Tin Resistance in Isonuclear Constructed Diploids

The isonuclear oligomycin and triethyl tin resistant diploids respond to triethyl tin on plates as shown in Table 3.1. The reaction of these diploids to oligomycin at the level of sub mitochondrial particles is shown in Fig. 3.16. The presence of the mutation conferring resistance to triethyl tin can be seen to confer a four fold increase in oligomycin resistance in the genotype $O^{S}T^{R}{}_{16}$. This level of resistance is the same as that observed in the strains carrying the mutation conferring resistance to oligomycin. Thus there is a phenotypic interaction between triethyl tin resistance and oligomycin resistance at the level of sub mitochondrial particles.

A further study was carried out to see if this interaction was retained after removal of the ATPase from the membrane or whether the interaction was lost due to removal of possible membrane constraints or separation of the peptide species expressing the interaction. As can be seen from Fig. 3.17 the differentials in oligomycin resistance between $O^{S}T^{R}$, $O^{R}T^{R}$, $O^{R}T^{S}$ and $O^{S}T^{S}$ are maintained and the interaction phenomenon is contained wholly within the ATPase complex.

In removing the ATPase complex from the membrane of the diploids the enzyme acquires a much higher sensitivity, in the region of ten fold higher, to oligomycin; this effect was also found with triethyl tin. This effect was not found with any of the haploids tested and indeed as can be seen from Figs. 3.16 and 3.17 ATPase from D22 included as a control strain maintains, very closely, its reaction to oligomycin during removal from the membrane. This effect may be a strain

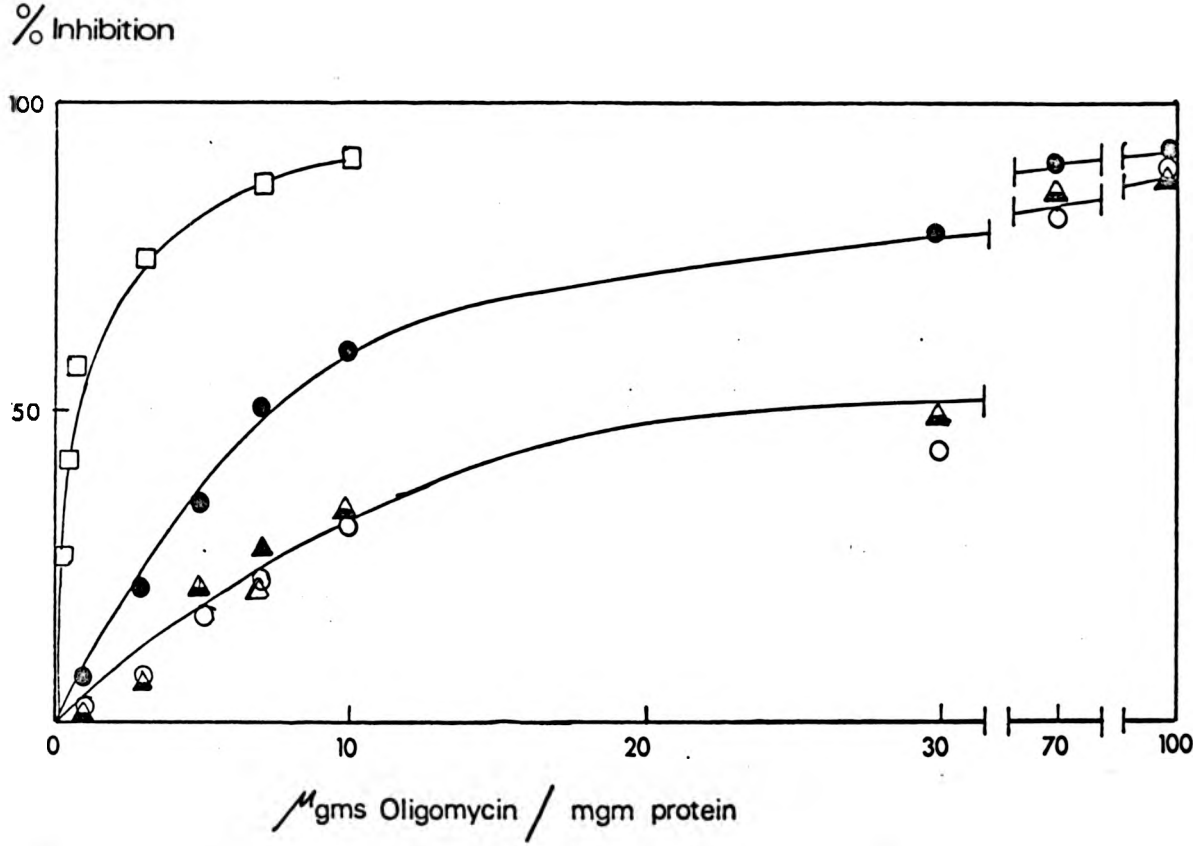
FIGURE 3.16

Oligomycin Sensitivity of Constructed Diploid Strains

SMP were prepared, as outlined in Materials and Methods from the following strains:-

| | | | |
|---|---|---|---|
| Diploid | KL114 | $O^{S}T^{R}$ | (●—●) |
| Diploid | KL111 | $O_{7}{}^{R}T_{16}{}^{R}$ | (△—△) |
| Diploid | KL110 | $O^{S}T_{16}{}^{R}$ | (○—○) |
| Diploid | KL113 | $O^{R}T^{S}$ | (▲—▲) |
| Haploid | D22 | $O^{S}T^{S}$ | (□—□) |

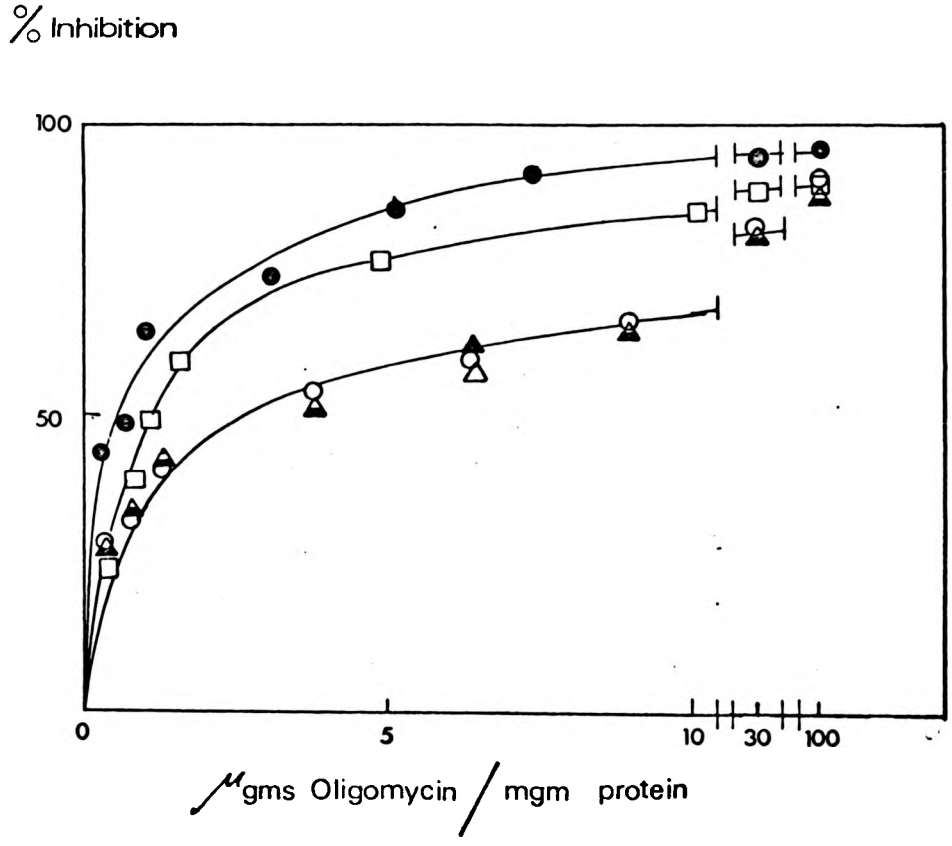
ATPase activity of the SMP was titrated against oligomycin, 100 $\mu$gm of protein was used per assay.


% Inhibition
100
50
0
10
20
30
70
100
μgms Oligomycin / mgm protein

ods

o-

FIGURE 3.17




Oligomycin Sensitivity of Triton Extracts from Constructed Diploid Strains

0.25% Triton X-100 extracts were prepared as outlined in Materials and Methods from the following strains;

Diploid KL114 $O^{S}T^{S}$ (●—●)

Diploid KL111 $O_{7}{}^{R}T_{16}{}^{R}$ (△—△)

Diploid KL110 $O^{S}T_{16}{}^{R}$ (○—○)

Diploid KL113 $O^{R}T^{S}$ (▲—▲)

Haploid D22 $O^{S}T^{S}$ (□—□)

ATPase activity of the Triton extract was titrated against oligomycin, approximately 30 μl of extract was used per assay.

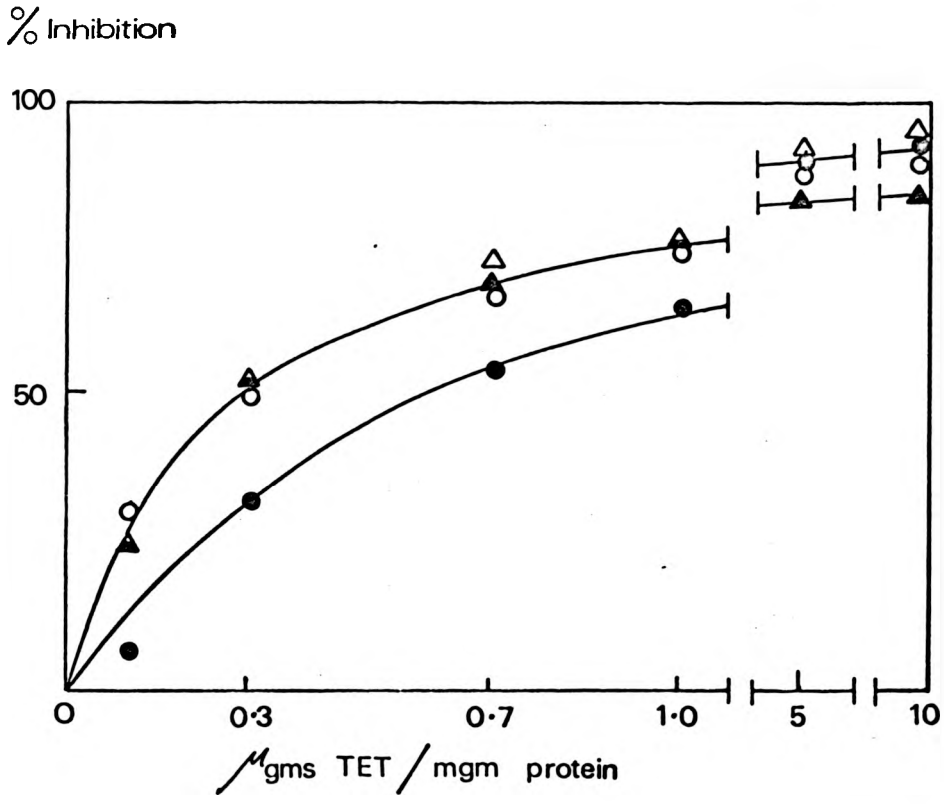
difference, as a similar effect has been reported by Somlo and Kruppa (1974).

An investigation was carried out to see if a converse interaction was taking place, i.e. was the presence of the mutation conferring oligomycin resistance affecting the response of the enzyme to triethyl tin. The strains $T^R$ marker became more sensitive to triethyl tin and venturicidin at the sub mitochondrial particle and soluble enzyme level by a factor of two as did the $O^RT^S$ genotype. Thus there would appear to be a converse interaction but of a more limited nature (Figs. 3.18 and 3.19).

## 3.4. DISCUSSION

Resistance to oligomycin has been demonstrated at the level of mitochondria in several cytoplasmically inherited oligomycin resistant mutants (Griffiths *et al.*, 1972; Azzone *et al.*, 1974; Houghton *et al.*, 1974). This study further demonstrates these resistances at the level of a crude, solubilised, membrane free ATPase preparation and at the level of the purified enzyme. Evidence is also provided which indicates that the mutational change expressed in the enzyme does not involve any great morphological change in the enzyme, this is as one would expect as the mutants had a full functional oxidative phosphorylation capacity, something which would not have been expected if gross deletions had occurred in the ATPase complex. The peptide analysis of the enzyme from several different mutants by polyacrylamide gel electrophoresis was complicated by several factors: 1) The size range of the polypeptides making up the enzyme complex was very broad thus the comparatively long periods of electrophoresis necessary for separation of the higher molecular

FIGURE 3.18




Triethyl Tin Sensitivity of Constructed Diploid Strains

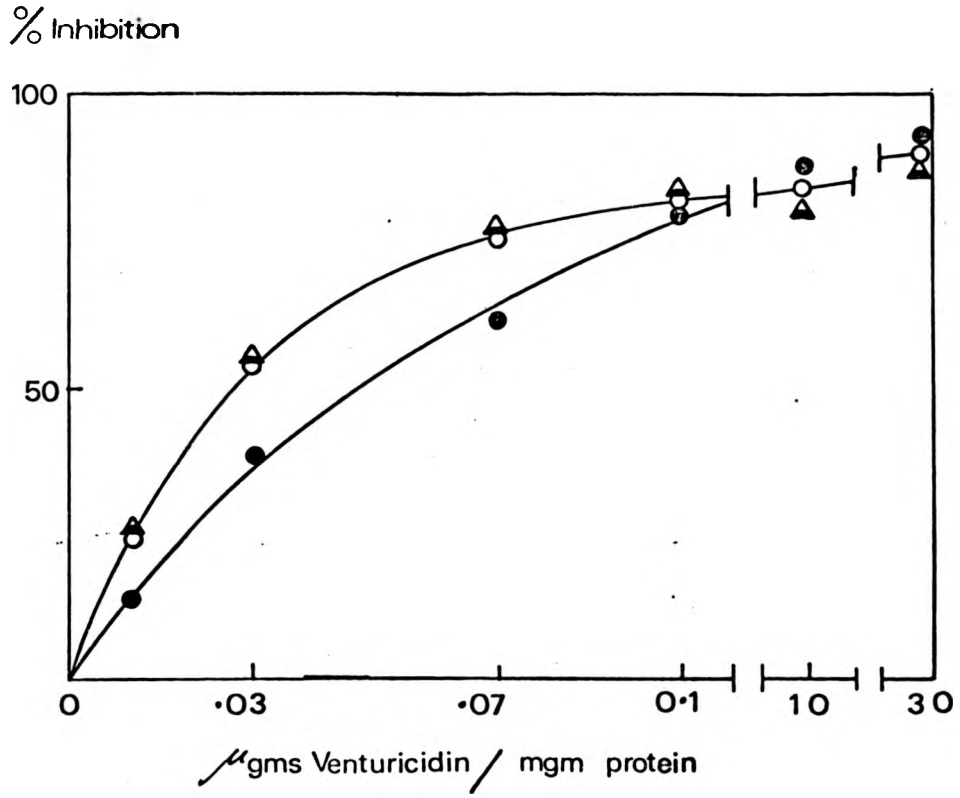
SMP were prepared as outlined in Materials and Methods from the following strains:

Diploid KL114 $O^{S}T^{S}$ (●——●) Diploid KL111 $O_{7}{}^{R}T_{16}{}^{R}$ (△——△)

Diploid KL110 $O^{S}T_{16}{}^{R}$ (o——o) Diploid KL113 $O_{7}{}^{R}T^{S}$ (▲——▲)

ATPase activity of SMP was titrated against TET, 100 μgms of protein was used per assay.

FIGURE 3.19




Venturicidin Sensitivity of Constructed Diploid Strains

SMP were prepared as described in Materials and Methods from the following strains:

Diploid KL114 $O^{S}T^{S}$ (●—●) Diploid KL111 $O_7{}^{R}T_{16}{}^{R}$ (△—△)

Diploid KL110 $O^{S}T_{16}{}^{R}$ (○—○) Diploid KL113 $O_7{}^{R}T^{S}$ (▲—▲)

ATPase activity of SMP was titrated against venturicidin, 100 μgms of protein were used per assay.

weight peptides resulted in some diffusion and subsequent zone broadening of the lower molecular weight species. Two approaches were adopted to attempt to overcome these problems; firstly a stacking gel was introduced into the gel system, a 7.5% acrylamide gel being poured on top of either 10%, 12.5% or 15% acrylamide gels. It was hoped that after separation from the high molecular weight peptides in the 7.5% gel the low molecular weight species would undergo zone sharpening on entering the more concentrated gel, however in all cases the protein was found to become immobilised at the surface of the second gel and in no way could this problem be overcome. A second attempt to overcome the problem was to use continuous gradients of acrylamide and so overcome the problem of sharp interfaces within the gel. However gradients built in this fashion, with a simple two chamber device were found to distort badly upon gel polymerisation. It is felt that with new analytical gel apparatus and precast gradient gels now becoming commercially available that better analyses should be obtainable.

2) The purified enzyme contains a relatively high proportion of lipid and this tends to streak through that part of the gel in which low molecular weight peptides are resolved. The lipid picks up stain and so completely obscures low molecular weight peptides. This problem was largely overcome by washing the enzyme with acetone to remove most of the lipid.

3) The low molecular weight, mitoribosomally synthesised peptides constitute a small proportion of the protein of the OS ATPase and subsequently their accurate analysis as part of the enzyme complex is rendered more difficult. It would be difficult to show that any enrichment procedure adopted for the mitoribosomal

products from the OS ATPase would be quantitative and so subsequent analysis of their stoichiometry would not be very meaningful. Thus it is felt that the analytical data provided here agrees well with that of Tzagoloff et al. (1973) and that the mutations do not lead to any gross changes in the peptide composition of the OS ATPase.

The enzyme has also been shown to be a site of action of triethyl tin and venturicidin, two inhibitors which do not affect $F_1$ ATPase and that the resistances to these two inhibitors conferred by mutation at locus OL111 (A19) are also expressed at the level of purified enzyme. The fact that the different levels of inhibitor resistance are maintained throughout the various stages of a preparation which involves gross changes in protein and lipid environment lends very strong support to the hypothesis that these mutations lead to altered protein binding sites on the ATPase complex.

Two suggestions present themselves from the data concerning changes in ATPase pH profiles between the wild type and mutant enzymes: 1) That a change in an ionising group is a result of the mutations at OL1 (A21) and OL111 (A19). If this is so, in view of the highly hydrophobic nature of the peptides making up the membrane factor and their subsequent low content of polar amino acids the amino acid change involved in such a mutation may be easier to detect than a mutation involving a change from one apolar amino acid to another. 2) The mutations at OL1 (A21) and OL111 map very closely together and are probably expressed in the same peptide, whilst OL11 (A15) maps at a completely different locus (Avner et al., 1973) and is presumably expressed in a different peptide. The studies on purified enzyme have shown that the phenotypic expression of OL1, OL111 and OL11 are

are located on the purified ATPase complex, it therefore seems reasonable to suppose that at least two mitochondrially coded peptides have the ability to confer oligomycin resistance. However the peptide expressing mutations OL1 and OL111 has a much greater effect on the enzyme activity at various pHs than that expressing OL11; it may be therefore that spacially and/or functionally the peptide expressing OL1 and OL111 is much closer to the ATPase active site of the enzyme than is the peptide expressing OL11.

The data obtained from the isonuclear constructed diploids concerning interactions between oligomycin and triethyl tin resistance suggests a close spatial and/or functional arrangement of the sites of action of the two inhibitors. The data on venturicidin inhibition and possible interactions is not as decisive as that with oligomycin but it can be tentatively suggested that venturicidin and triethyl tin resistance are affected in the same manner and to the same extent by inclusion of the mutation conferring oligomycin resistance. This may be a very tentative indication that triethyl tin and venturicidin have the same or very similar site of action. These results indicate, all be it indirectly, for the first time, that a mutation conferring TET resistance in vivo does affect in vitro inhibitor resistances.

It is of interest to note that while inclusion of the $T^R$ marker markedly increases the resistance of the diploid to oligomycin the inclusion of an $O^R$ marker does not further increase this resistance, while with the converse of this situation with the effect of $O^R$ markers on TET resistance on plates inclusion of the $T^R$ marker further increases the resistance to TET. Possible explanations for this phenomenon are:-

1) That the cell does not become more resistant to oligomycin and when the $T^R$ marker is inserted into the diploid it fully depresses the oligomycin resistance to a basal level.
2) That the assay system and/or inhibitor concentrations used do not reflect the possible attainable levels of oligomycin resistance.

# CHAPTER 4

## KINETICS OF BIOGENESIS OF INHIBITOR SENSITIVITY IN THE MITOCHONDRIAL ATPase OF STRAINS D22, D22A19 and D22A21.

### 4.1 INTRODUCTION

This study was undertaken with a view to the possible identification of the mutated mitochondrially coded and synthesised subunit(s) responsible for the decrease in sensitivity to oligomycin, in the case of D22A21, and to oligomycin, triethyl tin and venturicidin in the case of D22A19.

The mutation leading to the resistance phenomena may give rise to several discernible changes in the mitochondrial membrane proteins such as (a) a change in rate of synthesis of the mutated subunit (b) a change in the temporal assembly of the ATPase complex within the inner mitochondrial membrane. Such an assembly effect has been postulated by Linnane (personal communication) to explain a low maximal inhibition of ATPase by oligomycin in an oligomycin resistant mutant. (c) A change in the amount of subunit(s) synthesised thus producing a change in the stoichiometry of the membrane and/or the ATPase complex.

Any of these changes may be expressed as a discernible change in inhibitor sensitivity during incorporation of insensitive $F_1$ ATPase into mitochondrial sensitive ATPase (Tzagoloff et al., 1972) and that by following the kinetics of this change and correlating it with subunit(s) synthesis one may be able to identify the aberrant subunit(s).

In yeast both $F_1$ and OSCP are made by the cytoplasmic

ribosomal protein synthesising system (Tzagoloff, 1969; 1970). This conclusion was based on observation that chloramphenicol a specific inhibitor of mitochondrial protein synthesis (Wintersberger, 1965; Lamb, et al., 1968) failed to inhibit the synthesis of $F_1$ and OSCP (Tzagoloff, 1969; 1970). The synthesis of these two components was however inhibited by cycloheximide, an inhibitor of cytoplasmic protein synthesis (Wintersberger, 1965; Lamb et al., 1968). Using the known site of action of these two inhibitors Tzagoloff was able to study the incorporation of $F_1$ ATPase subunits into the oligomycin sensitive ATPase complex during synthesis of the membrane factor. The use of cycloheximide during derepression allows specific radioactive labelling of mitochondrially synthesised proteins. However the increase in the membrane factor if the cells are derepressed in the presence of cycloheximide was negligible (Tzagoloff, 1971). However if the cells are preincubated in chloramphenicol, i.e. cytoribosomal activity is allowed to proceed, then during derepression in the presence of cycloheximide, membrane factor activity was increased two to three fold and subsequent incorporation of $F_1$ ATPase into the inner mitochondrial membrane may be monitored.

Thus the procedure of sequential poisoning suggested itself as a suitable system with which to monitor:-

1) Production of mitochondrially synthesised peptide(s) responsible for ATPase inhibitor sensitivity or resistance.

11) Possible stoichiometric and/or temporal interactions between mitochondrially synthesised membrane peptides and cytoplasmically synthesised $F_1$ ATPase resulting in inhibitor sensitive or resistant membrane bound ATPase complex.

In order to positively identify any mitoribosomal products as OS ATPase, subunits specific antisera to OS ATPase were raised and tested on Ouchterlony plates using preimmune sera, and purified cytochrome oxidase to display the specificity of the antisera for OS ATPase.

It was important to show that any increases in ATPase activity or changes in inhibitor sensitivity were due to incorporation of new enzyme into the inner mitochondrial membrane and were not due to other phenomena such as the unmasking of latent activity and sensitivity by some unidentified mitoribosomal product. To this end the increase in inner membrane OS ATPase was monitored in several ways:- 1) The increase in inhibitor sensitive activity in SMP was correlated with the disappearance of aurovertin and Dio-9 sensitive activity from the PRS during derepression in CHI. 2) Analysis of inner mitochondrial membrane proteins by acrylamide gel electrophoresis to monitor the increases of high molecular weight components associated with the OS ATPase in the membrane. 3) The increase in extractable OS ATPase as analysed by gradient centrifugation, during derepression in CHI was monitored.

Changes in OS ATPase activity or sensitivity could be brought about by working with a mixed population of petite and grande cells. In the light of reports stating that prolonged exposure to CAP induces petites at an abnormally high rate it was thought necessary to check the cells for petite frequency after incubation in CAP.

It was felt necessary to demonstrate that the counts incorporated in the presence of CHI were CAP sensitive i.e. were incorporated by the mitoribosomal system and did not merely represent a 5% CHI resistant cytoribosomal incorporation.

## 4.2 METHODS AND MATERIALS

### Growth of S. cerevisiae

S. cerevisiae was grown aerobically in 4 ltr. cultures in the dark at 30°C. Compressed air was passed through a sparger into the culture at a rate of 1 ltr of air/min/ltr of culture. The culture being stirred magnetically at a rate of 150 r.p.m. The medium contained per litre of distilled water:- 1 gm $KH_2PO_4$, 1.2 gm $(NH_4)_2SO_4$, 0.5 gm NaCl, 0.7 gm $MgSO_4.7H_2O$, 0.4 gm $CaCl_2$, 0.005 gm $FeCl_3$, 0.1 gm adenine sulphate, 0.3% w/v yeast extract. Tributyl citrate at a concentration of 0.2 ml/ltr was used as an antifoaming agent. Glucose was used as a carbon source at the concentrations indicated and was added as a sterile 40% solution after autoclaving.

A 1% v/v inoculum from a starter culture grown to stationary phase on 0.8% glucose was inoculated into 4 ltr of medium containing 5.4% glucose. The cells were harvested in early stationary phase and transferred to fresh medium containing 0.8% glucose and 2 mg/ml of chloramphenicol. After 10 hours aeration the cells were harvested, washed in cold sterile distilled water and inoculated into fresh medium containing 0.8% glucose and $5 \times 10^{-5}$ cycloheximide. One fifth to one quarter of this culture was harvested at the various time intervals indicated; chloramphenicol being added to the harvested culture to prevent respiratory adaptation taking place during processing.

### Preparation of Sub mitochondrial Particles

These were prepared from Braun shaken mitochondria, the

procedure is exactly the same as that described in chapter 2.

Assay of ATPase Activity and Sensitivity

The assay was carried out as described previously in Chapter 2.

Preparation of Post-ribosomal Supernatants

The supernatant of the post mitochondrial fraction was spun at 50,000r.p.m. for 1 hour in a Beckman 50 Ti rotor, the resulting supernatant was collected and designated the post-ribosomal supernatant (PRS).

Demonstration of CAP Sensitivity of $C^{14}$-leucine Incorporated in the Presence of CHI

D22 was grown to early stationary phase and harvested, washed and resuspended in sterile water at a concentration of 40 mg wet weight of cells/ml. The procedure was carried out as aptically wherever possible, no bacterial contamination was visible under the microscope. 0.5 ml of cells were added to 0.5 ml of a solution containing a 2 fold concentration of growth medium and 1.6% glucose. The cells were preincubated for 30 min with the inhibitors indicated and then inoculated with 2$\mu$c of $C^{14}$-leucine and shaken for 3 hours at 30°C. The cells were killed by the addition of 1 ml of ice cold 10% TCA and filtered through Whatman GF/A fibreglass filters. The filters were washed sequentially with two 5 ml aliquots of 10% TCA and three 5 ml aliquots of absolute ethanol. No counts were recovered in the final wash. The filters were dried and placed directly in 25 ml vials and counted after the addition of 10 ml of scintillant (0.5l Triton X-100, 1.0l toluene, 7 gm

butyl PBD).

Radioactive Labelling of Mitochondrial Synthesis Products

The conditions for the growth of yeast were identical to those described previously (see growth of S. cerevisiae, this Chapter) except that 500 ml of culture was used and in the final phase of aeration in the presence of $5 \times 10^{-5}$M cycloheximide the medium was supplemented with 14 µci/ml of L-leucine 4,5- $^{3}H$ with a specific activity of 58 ci/mmole. The leucine was added as a 2% aqueous solution of ethanol at a concentration of 1 mci/ml. The cells were harvested and poisoned with 2 mg/ml chloramphenicol at the time intervals indicated.

Preparation of Labelled Sub mitochondrial Particles

The procedure for preparing labelled sub mitochondrial particles was exactly the same as described in Chapter 2 except that the yeast suspension concentration was cut from 10 gm wet weight of cells/30 ml MTE to 2 - 5 gm wet weight cells/30 ml and sonication of the mitochondria was carried out in an MSE 50 Kc sonic oscillator for 30 seconds on maximum setting.

Preparation of Triton Extracts

Yeast sub mitochondrial particles were suspended in 5 mM Tris acetate pH 7.5 at a final protein concentration of 6.4 mg/ml. To this was added a 10% w/v solution of Triton X-100 in 5 mM Tris acetate pH 7.5 to a final concentration of 0.25% except where indicated in the text. The mixture was centrifuged at 105,000 g for 20 minutes. The clear infranatant was taken discarding the pellet and lipid laden supernatant.

Where complete removal of ATPase from the membrane was required and the concentration of Triton was not critical 0.5% w/v of the detergent was used as shown in Fig. 3.2; this should give virtually complete removal of ATPase from the membrane.

Preparation of Antiserum to Oligomycin Sensitive ATPase

Oligomycin sensitive ATPase was prepared from bakers yeast as described in Chapter 3. The final density gradient centrifugation was carried out in an MSE 6 x 300 ml angle rotor, 25 ml of triton extract being layered onto a 250 ml, 5 - 20% sucrose gradient containing 5 mM Tris acetate pH 7.5 and 0.1% w/v Triton X-100. The gradients were spun at 19,000 r.p.m. for 24 hours and fractionated, the active fractions were pooled and concentrated to 7 mg/ml in an Amicon ultrafilter over a diaflow XM100 membrane. 1 ml of enzyme was thoroughly homogenised with an equal volume of Freunds complete adjuvant. After an initial bleeding to obtain control serum this mixture was injected into an adult rabbit in three equal doses, two intramuscularly into the hind legs and one subcutaneously in the neck region. This procedure was repeated three weeks later with a further 1 ml of enzyme homogenised with an equal volume of Freunds complete adjuvant. Approximately 20 ml samples of blood were collected starting one week after the second injection, from the lateral vein of the ear. The blood was allowed to clot for 1 hour at room temperature and a glass rod passed carefully around the clot freeing it from the wall of the test tube and allowing it to contract for a further hour. The yellowish serum was removed with a Pasteur

pipette. The clot was cut into small pieces in a small glass beaker, sealed with parafilm and left overnight at 0°C. The mixture was spun in a bench centrifuge at 3,000 r.p.m. for 15 minutes and the serum supernatant collected. The antisera was stored at -20°C in small aliquots and remained active for at least two years.

Antisera to cytochrome oxidase antigen, prepared by the method of Rubin and Tzagoloff (1973) was prepared in a similar manner.

Preparation of Double Diffusion Plates

Preparation of Agar 15 gm of Nobles special agar (Difco) was washed 4 times in 1 ltr of distilled water and the fines removed. 8 gm of NaCl and 1 gm of sodium azide were dissolved in distilled water and the washed agar and water added to a final volume of 1 ltr. The final concentration of agar is about 1.2%, 0.3% being lost in washing.

The agar was dissolved by heating at 90°C in a water bath with constant stirring. The molten agar was divided into 7 ml lots in screw capped vials and stored at 4°C. The agar was found to keep indefinitely if the vials were well sealed.

Preparation of Plates 7 ml of molten agar (1 vial) was poured onto a clean plastic petri dish on a levelling table. While still molten the agar surface was flamed to prevent bubble formation. When the agar was set holes were punched as required with a number 2 cork borer and the agar removed with a Pasteur pipette attached to a water pump. The wells were filled with the required amount of antibody or antigen. The filled plate was placed in an airtight box lined with damp cloth and

incubated at 4°C for 96 hours. Although precipitin lines formed more quickly at room temperature it was found that better resolution was obtained at 4°C.

Staining of Plate The plate was washed for 30 hours with two changes of buffered saline (50 mM $Na_2HPO_4$, 0.8% NaCl pH 7.0) to elute out uncomplexed proteins. The precipitated proteins are then stained by immersion in a solution of 0.05% Coomasie Blue in water; acetic acid; methanol (5:1:5 v/v) for 10 minutes and destained in several changes of destain (Water: acetic acid: methanol, 5:1:5 v/v).

Preparation of Samples for Disc Gel Electrophoresis

An aliquot of a suspension of SMP containing 1 mg of protein was diluted to a final volume of 0.34 ml with 5 mM Tris and a 1 M KCl solution was added to give a final concentration of 4 mM. 4 volumes of acetone were added and the mixture was vigorously shaken in a glass, conical test tube and kept on ice overnight. The precipitate was spun down in a bench centrifuge at 3,000 r.p.m. for 5 minutes. 1 ml of a solution containing 1% SDS, 1% mercaptoethanol, 10 mM $PO_4$, 10% glycerol and 3 µl of a 0.005% aqueous bromophenol blue solution was added to the acetone pellet which was broken up with a glass rod. The mixture was boiled for 1 minute and heated at 70°C for 20 minutes, cooled and applied to a 10 cm 7.5% acrylamide gel of 5 mm diameter and electrophoresed for approximately four hours at a constant current of 8 milliamps/gel. The procedure for staining and destaining the gels was as described in Chapter 3.

Preparation of Tritiated Samples for Disc Gel Electrophoresis

An aliquot of tritiated SMP at a protein concentration of

2 mg/ml was dissolved in an equal volume of a solution containing 2% SDS, 2% mercaptoethanol, 20 mM $PO_4$, 20% glycerol and 3 µl of a 0.005 aqueous bromophenol blue solution. The mixture was boiled for 1 minute and heated at 70°C for 20 minutes, cooled and applied to the gel.

Preparation of Antibody/Antigen Precipitate for Disc Gel Electrophoresis

A 0.5% Triton extract prepared from tritiated SMP was divided into two equal volumes, to one was added an equal volume of antisera to oligomycin sensitive ATPase and to the other was added an equal volume of control, preimmune sera. Both samples were incubated for one hour at room temperature and subsequently kept at 0°C overnight. Only the sample containing the Triton extract, antisera mix contained a precipitate, no visible precipitate being formed with the control, preimmune sera. The antibody-antigen precipitate was dissolved directly in 100 µl of a solution containing 1% SDS, 1% mercaptoethanol, 10 mM $PO_4$, 10% glycerol and 3 µl of a 0.005% aqueous bromophenol blue solution.

Disc Gel Electrophoresis for Staining

7.5% acrylamide gels measuring 6 mm diameter by 100 mm length were prepared by the method of Weber and Osborn (1969).

100 µg of the acetone precipitated SMP samples were applied to each gel, to a control gel was applied 80 µg of purified oligomycin sensitive ATPase. The gels were run at 8 mamps/gel for approximately 4 hours and stained and destained as described in Chapter 3.

Disc Gel Electrophoresis of Tritiated Samples

Initially 100 µg tritiated samples were run on 7.5% acrylamide gels of 6 mm diameter however in view of the low incorporation of tritium into some of the samples, insufficient counts to give rise to definite peaks of activity could be put onto the gel. To overcome this problem gels of 14 mm diameter were cast and 400 µg of samples applied without undue band broadening. The gels were run at 20 milliamps per gel for approximately 5 hours. The gels were stored at -20°C overnight and sliced into 1.1 mm thick slices on a Mickle gel slicer (Mickle Scientific Instruments, Carshalton, Surrey) and digested with 0.4 ml of 6% hydrogen peroxide in tightly capped vials at 70°C for 17 hours. A further 0.6 ml of water was added to prevent the production of unstable emulsions on addition of the scintillant. 10 ml of scintillant (1 ltr Toluene, 0.5 ltr Triton X-100, 7 gm of butyl PBD) was added to each vial and the samples counted on a PacKhard 5000 scintillation counter.

Testing for Petite Frequency in CAP Incubated Cells

Cells which had been incubated for 10 hours in CAP were spread on plates containing 1% yeast extract, 1% peptone, 40 mM $KH_2PO_4$, 10 mM $Na_2HPO_4$, 2.3% agar, 0.1% glucose and 3% glycerol. The cells were spread at a density of 100/plate and incubated at 30°C for 3 days and scored for either petite or grande colony formation. The frequency of petite induction in these cells showed no appreciable difference from cells not incubated in CAP.

## 4.3 RESULTS

The yield of SMP protein fell slightly throughout the incubation period in CHI while the post ribosomal supernatant after an initial rise during the first hour fell back to its original level after 3 hours as shown in Table 4.1. Table 4.2 and Fig. 4.1 monitor the changing ATPase activities of the SMP and post ribosomal supernatants. Table 4.2 shows that SMP ATPase activity more than doubles after3 hours incubation in CHI and that this increased activity is susceptible to inhibition by oligomycin and venturicidin. Fig. 4.1 correlates the removal of a Dio-9, aurovertin sensitive ATPase activity, presumably $F_1$ATPase from the PRS with the concommitant rise in venturicidin sensitive SMP ATPase activity. Figs. 4.2 and 4.3 demonstrate that the increase in SMP ATPase activity is due to an incorporation of new sensitive enzyme and is not due to an unmasking of latent activity or sensitivity. The incorporation of the high molecular weight peptides associated with the OS ATPase appears to be essentially complete after three hours incubation in CHI.

The ATPase activity of the wild type cells grown in CAP for 10 hours is sensitive to the inhibitors oligomycin, venturicidin and triethyl tin. The sensitivity displayed by newly incorporated enzyme shows no significant variation from the enzyme in the original CAP cells, i.e. 0 hour samples (Figs. 4.4, 4.5 and 4.6). The same is substantially true of A19 cells (Figs. 4.7 and 4.8), with a small but hardly significant decrease in sensitivity to oligomycin on growth in CHI. The enzyme sensitivity of the mutant A21 shows a much lowered sensitivity and maximum attainable inhibition after

TABLE 4.1

Protein Recoveries During Derepression in Cycloheximide

| Hours in CHI | G wet weight cells/100 ml media | Mg SMP protein /g wet weight cells | Mg PRS protein /g wet weight cells |
|---|---|---|---|
| 0 | 2.84 | 1.10 | 17.29 |
| 1 | 2.3 | 0.97 | 20.0 |
| 3 | 3.0 | 0.86 | 17.3 |

TABLE 4.2

Increase in ATPase Activity During Derepression in CHI

| Hours in CHI | Hours 0 | 1 | 3 |
|---|---|---|---|
| Total SMP ATPase activity | 1.38 | 2.0 | 3.0 |
| SMP ATPase activity inhibited by 100 µg oligomycin/mg protein | 1.32 | 1.8 | 2.72 |
| SMP ATPase activity inhibited by 5 µg Ven/mg protein | 0.92 | 1.8 | 2.72 |

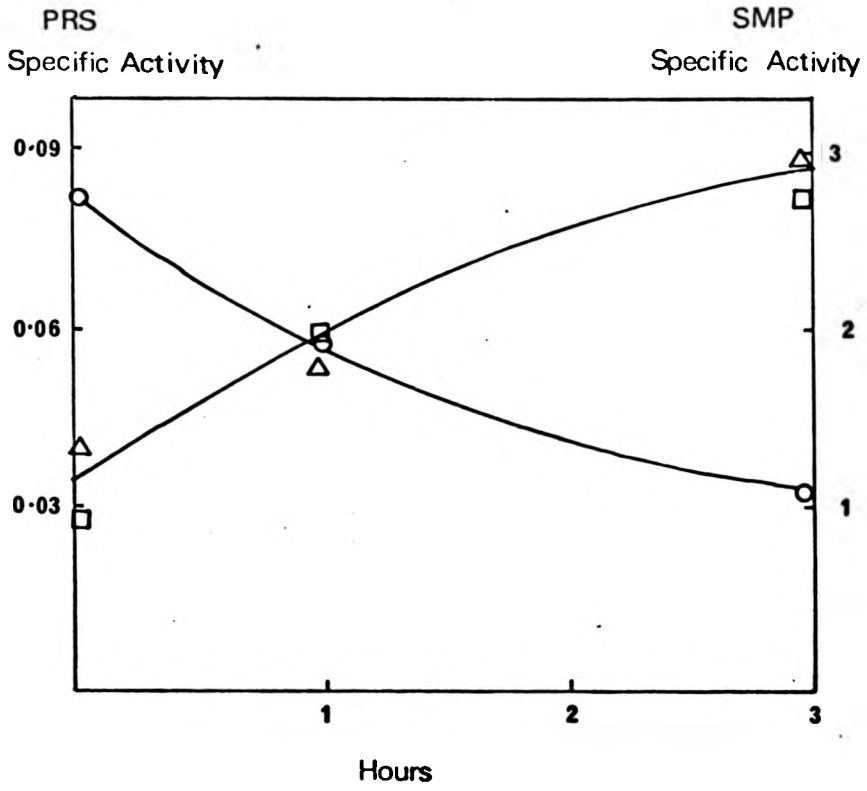


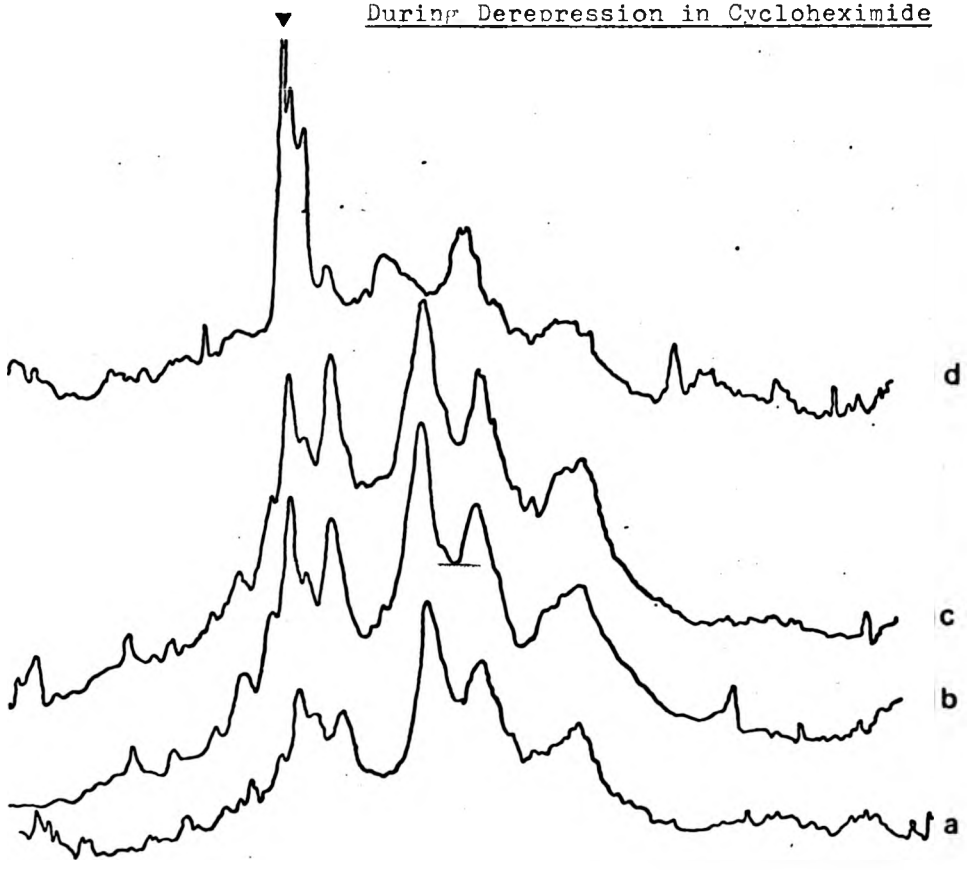

FIGURE 4.1

Changes in ATPase Activity of Post Ribosomal Supernatant and Sub Mitochondrial Particles During Derepression in Cycloheximide

SMP and PRS were prepared as described in Materials and Methods. The PRS was assayed in the presence of Dio-9 and aurovertin and only activity sensitive to these inhibitors is shown (o—o). SMP total ATPase activity (Δ—Δ) and SMP ATPase activity sensitive to 5 μgm venturicidin/mg protein are shown (□—□).

FIGURE 4.2

Changes in Protein Profiles of Sub Mitochondrial Particles During Derepression in Cycloheximide

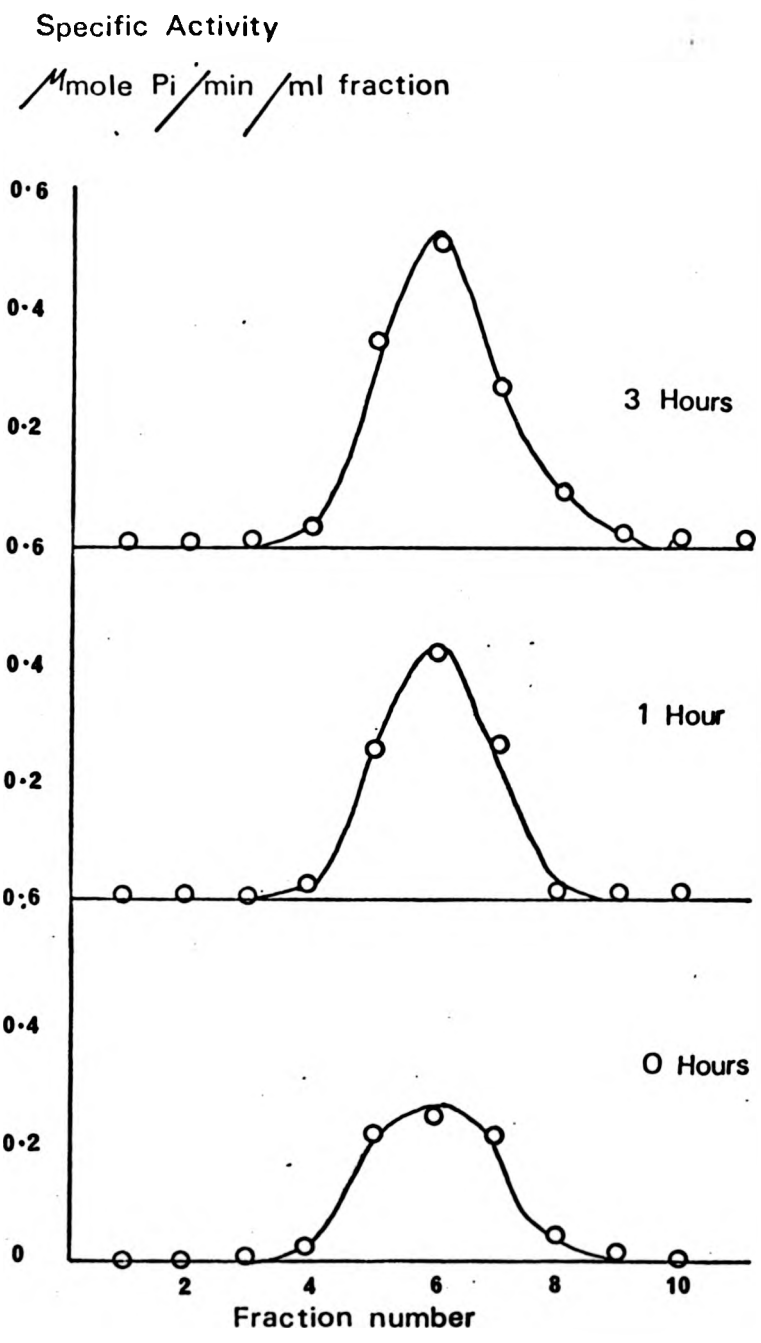



Samples for electrophoresis were prepared as described in Materials and Methods. 100 µg of protein of SMP prepared from cells derepressed in CHI for 0 hours (a); 1 hour (b); 3 hours (c); and 100 µg of OS ATPase were electrophoresed in 7.5% acrylamide gels at 8 milliamps/gel. The gels were stained and destained and scanned at 600nm in a Gilford Spectrophotometer with a scanning attachment. The position of the high molecular weight peptides of OS ATPase are marked (▼).

FIGURE 4.3

Changes in Levels of Extractable OS ATPase During Derepression in Cycloheximide

5 mg of SMP prepared from cells derepressed in CHI for 0, 1 and 3 hours were extracted with 0.5% w/v Triton X-100 in a total volume of 1 ml. This suspension was centrifuged at 105,000 r.p.m. for 20 minutes, the supernatants were removed and 0.5 ml of each layered onto a 5 ml continuous 5 - 20% sucrose gradient containing 10 mM Tris acetate pH 7.5 and 0.05% Triton X-100. The gradients were centrifuged for 4.5 hours at 40,000 r.p.m. in a Beckman SW 50 rotor and fractionated into ten equal fractions and assayed for ATPase activity.


Specific Activity
μmole Pi / min / ml fraction
0·6
0·4
0·2
0·6
3 Hours
0·4
0·2
0:6
1 Hour
0·4
0·2
0
O Hours
2
4
6
8
10
Fraction number

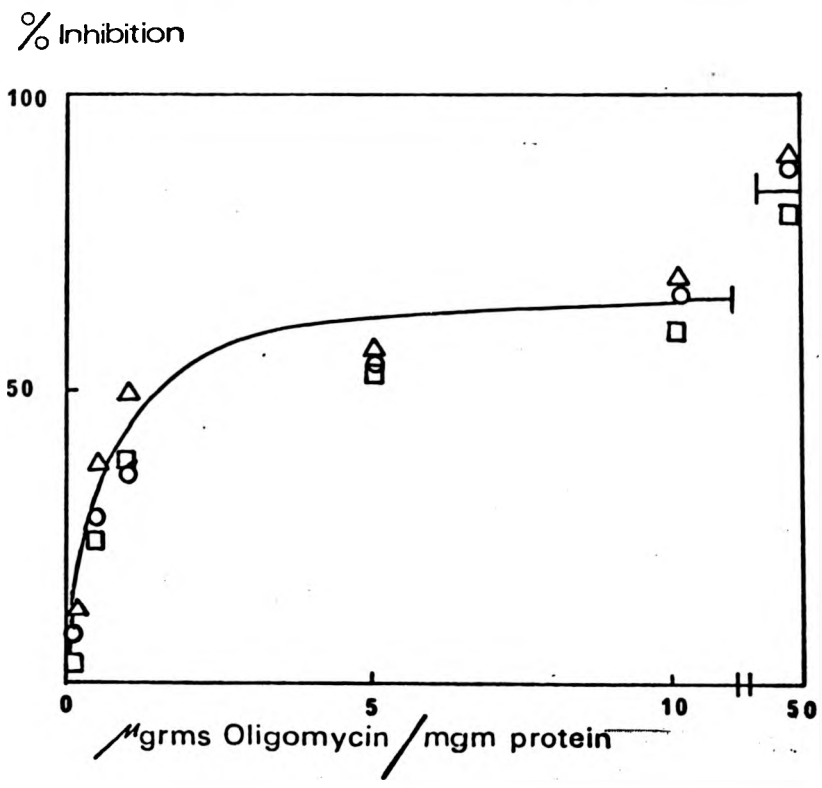



FIGURE 4.4

The Sensitivity to Oligomycin of D22 Sub Mitochondrial Particles from Cells Derepressed in Cycloheximide

Wild type cells were grown for 10 hours in CAP, washed and derepressed in the presence of CHI as described in Materials and Methods. SMP were prepared from cells after 0 hours (o——o); 1 hour (□—□); and 3 hours (Δ—Δ) in CHI and assayed for ATPase sensitivity to oligomycin.

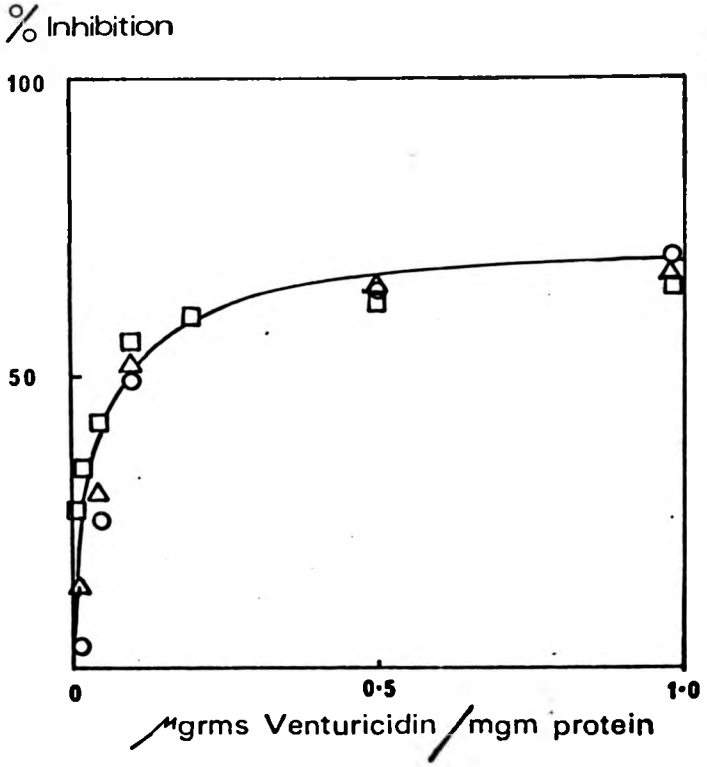



FIGURE 4.5

The Sensitivity to Venturicidin of Sub Mitochondrial Particles Prepared from D22 Cells Derepressed in Cycloheximide

Wild type cells were grown for 10 hours in the presence of CAP, washed and derepressed in the presence of CHI as described in Materials and Methods. SMP were prepared from cells harvested after 0 hours (o—o); 1 hour (□—□); and 3 hours (Δ—Δ) in CHI and the ATPase sensitivity to oligomycin was assayed.

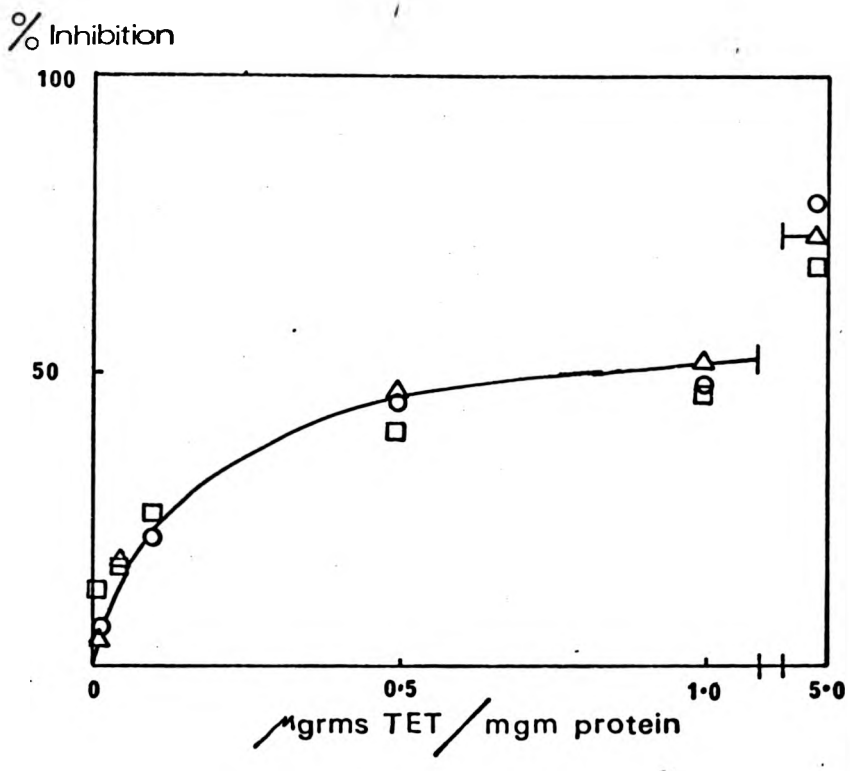



FIGURE 4.6

The Sensitivity to Triethyl Tin of Sub Mitochondrial Particles Prepared from D22 Cells Derepressed in Cycloheximide

Wild type cells were grown and treated as described in the legend to Fig. 4.4. Sub mitochondrial particles prepared from cells harvested after 0 hours (o—o); 1 hour (□—□); and 3 hours (Δ—Δ) in CHI were assayed for ATPase sensitivity to TET.

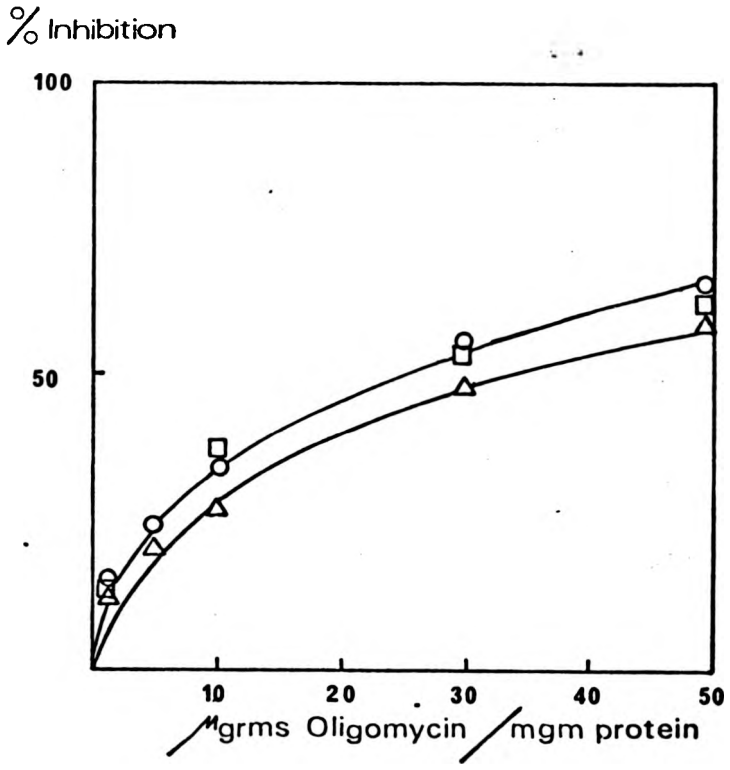



FIGURE 4.7

The Sensitivity to Oligomycin of Sub Mitochondrial Particles Prepared from A19 Cells Derepressed in the Presence of Cycloheximide

A19 cells were grown and treated as described in the legend to Fig. 4.4. Sub mitochondrial particles prepared from cells harvested after 0 hours (o—o); 1 hour (□—□); and 3 hours (Δ—Δ) in CHI were assayed for ATPase activity sensitive to oligomycin.

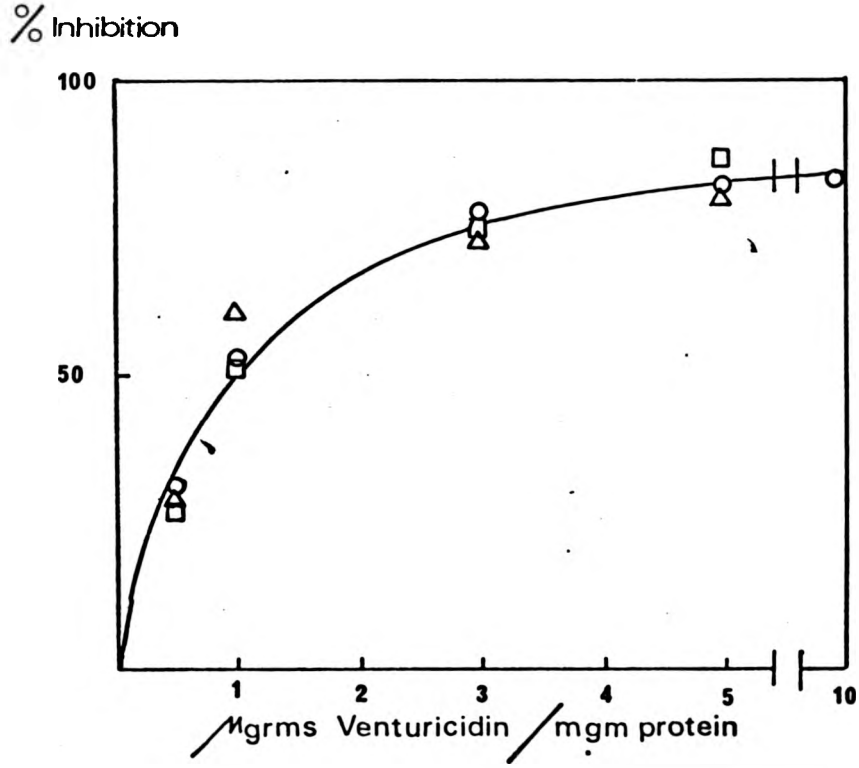



FIGURE 4.8

The Sensitivity to Venturicidin of Sub Mitochondrial Particles Prepared from A19 Cells Derepressed in Cycloheximide

A19 cells were grown and treated as described in the legend to Fig. 4.4. Sub mitochondrial particles prepared from cells harvested after 0 hours (o—o); 1 hour (□—□) and 3 hours (△—△) in CHI were assayed for ATPase sensitivity to venturicidin.

10 hours growth in CAP (Fig. 4.9). However after transference to 0.8% glucose medium supplemented with CHI the enzyme rapidly attains the normal level of sensitivity expected with this mutant, the process being essentially complete after 1 hour. This phenomenon does not appear to be restricted to the single inhibitor oligomycin against which the resistant mutant was selected, but also occurs with venturicidin and triethyl tin (Figs. 4.10 and 4.11) towards which A21 enzyme normally displays wild type levels of sensitivity. Two possible explanations of this behaviour present themselves. 1) That the change in sensitivity is an artifact brought about in some undefined, direct or indirect manner as a consequence of poisoning with CAP, this may lead to an alteration in the stereochemistry of the already assembled complex. 11) That during the initial growth in the repressing media the enzyme is being faultily assembled. Such a phenomenon should not occur during growth in CAP because of the lack of production of membrane factor for incorporating $F_1$ ATPase into the mitochondrial membrane.

In the light of these results it was decided to attempt an analysis of the mitoribosomal product during growth in CHI in wild type and A21 cells. A major problem in this investigation was that while keeping the amount of $^3$H-leucine required for the experiment to a reasonable level, not enough label could be introduced into the mitoribosomal products to enable a reasonable analysis of the peptide produced in the first hour of the growth in CHI to be carried out. Only after one hour was there sufficient incorporation to allow analysis of the peptide to proceed.

Table 4.3 demonstrates that 70% of the CHI resistant

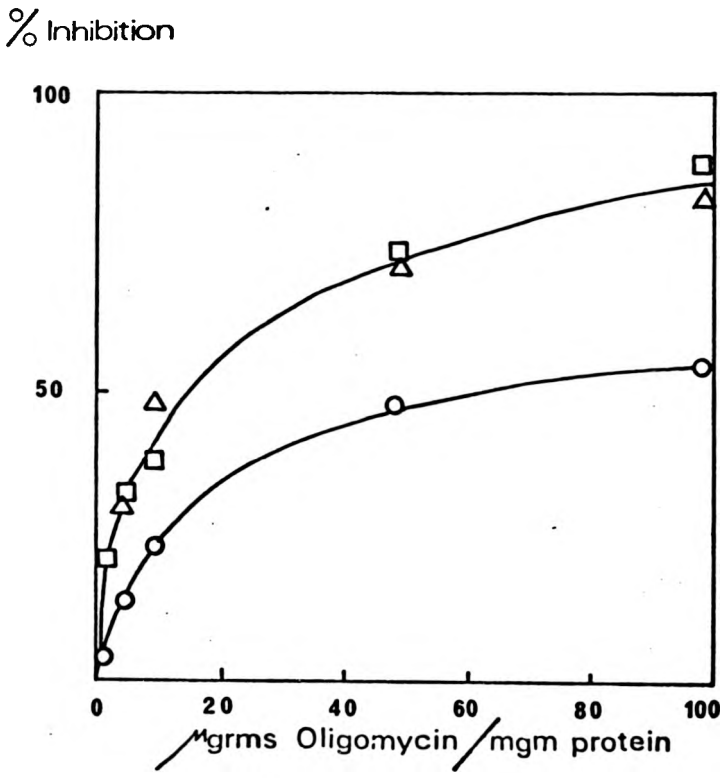



FIGURE 4.9

The Sensitivityto Oligomycin of Sub Mitochondrial Particles Prepared from A21 Cells Derepressed in Cycloheximide

A21 cells were grown and treated as described in the legend to Fig. 4.4. Sub mitochondrial particles prepared from cells harvested after 0 hours (o—o); 1 hour (□—□); and 3 hours (Δ—Δ) in CHI were assayed for ATPase sensitivity to oligomycin.

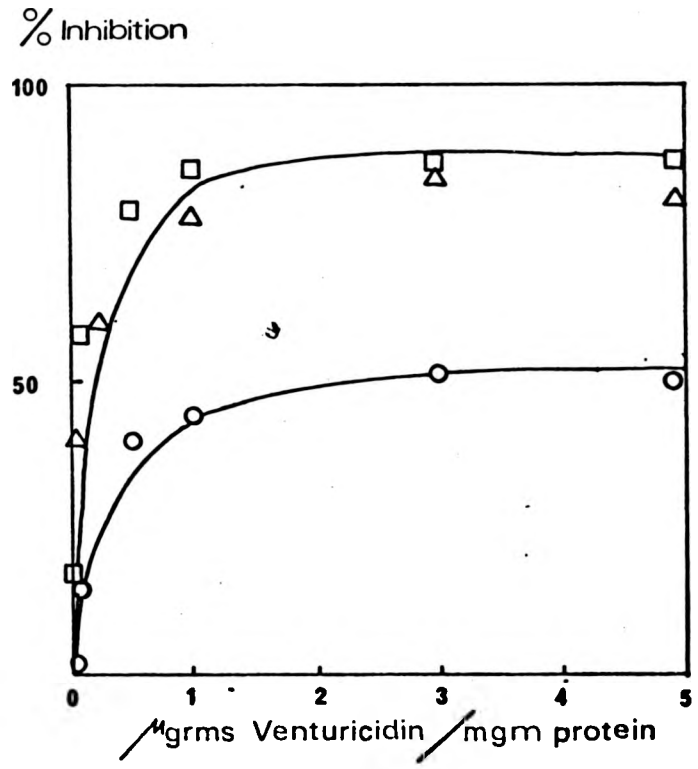



FIGURE 4.10

The Sensitivity to Venturicidin of Sun Mitochondrial Particles Prepared from A21 Cells Derepressed in Cycloheximide

A21 cells were grown and treated as described in the legend to Fig. 4.4. Sub-mitochondrial particles prepared from cells harvested after 0 hours (o——o); 1 hour (□—□); and 3 hours (Δ—Δ) in CHI were assayed for ATPase sensitivity to venturicidin.

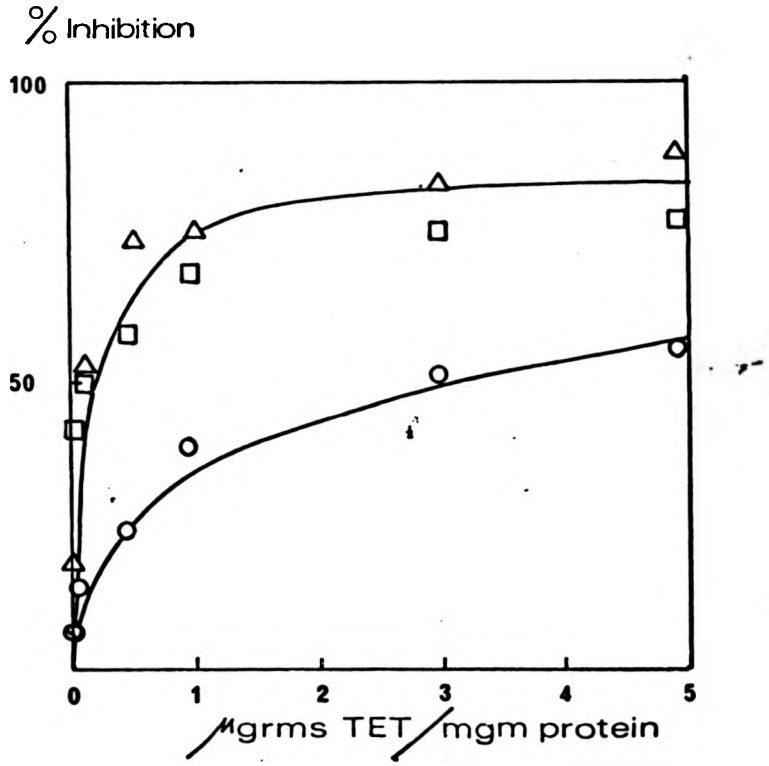



FIGURE 4.11

The Sensitivity to Triethyl Tin of Sub Mitochondrial Particles Prepared from A21 Cells Derepressed in Cycloheximide

A21 cells were grown and treated as described in the legend to Fig. 4.4. Sub mitochondrial particles prepared from cells harvested after 0 hours (o—o); 1 hour (□—□); and 3 hours (Δ—Δ) in CHI were assayed for ATPase sensitivity to TET.

TABLE 4.3

Chloramphenicol Sensitivity of Cycloheximide Resistant Counts

| Cycloheximide μg/ml | Chloramphenicol mg/ml | TCA precipitated c.p.m. |
|---|---|---|
| 0 | 0 | 92583 |
| 0 | 2 | 98275 |
| 0 | 4 | 90744 |
| 25 | 0 | 4355 |
| 25 | 2 | 1337 |
| 25 | 4 | 1218 |

incorporation was sensitive to CAP. A figure in good agreement with that obtained by England and Attardi (1974) in their work on Hela cells using emetine as a cytoribosomal inhibitor. The kinetics of incorporation into mitoribosomal products of wild type and A21 cells are very similar, as shown in Table 4.4. Incorporation into OS ATPase antibody precipitatable material of wild type and A21 cells are very similar (Table 4.5). The kinetics of incorporation showed no great differences in the mutant and wild type so the distribution of label within the mitoribosomal products was investigated.

After 1 hour incubation in CHI supplemented with $^{3}$H-leucine the major mitoribosomal product is a low molecular weight peptide( which moves ahead of the bromophenol blue dye and cytochrome c (Fig. 4.12). Such a product has been reported as the major mitoribosomal product associated with the OS ATPase. At least three other peaks appear on the gel. In view of the low level of incorporation and recovery of counts from the gel it is possible to state only tentatively that the incorporation patterns are similar. Figs. 4.13 and 4.14, show the mitoribosomal products after 3 hours and 6 hours respectively. The products after three hours do not seem to form a continuum of those shown at 1 hour and 6 hours and this may be an artifact. After 6 hours growth however the $^{3}$H-leucine is much more evenly distributed between the low molecular weight peak and a much higher molecular weight peak, but again the mutant and wild type patterns are very similar. Ebner *et al.*, (1973) report, in contradiction to Tzagoloff (1973) and the findings here , that they could find no evidence of a low molecular weight mitoribosomal product. They further report

TABLE 4.4

Kinetics of Incorporation of $^{3}$H-Leucine into Sub Mitochondrial Particles of D22 and A21 Cells

| Time in CHI (hours) | Cpm/mg incorporated into D22 SMP | Cpm/mg incorporated into A21 SMP |
|---|---|---|
| 0 | 4230 | 2866 |
| 1 | 11170 | 10730 |
| 3 | 29741 | 22118 |
| 6 | 35428 | 30866 |

Cells were grown and SMP prepared as described in Materials and Methods.

TABLE 4.5

Kinetics of Incorporation of $^{3}$H-Leucine into Antisera Precipitable Components of OS ATPase

| Time in CHI | Total cpm in D22 antibody precipitate | Total cpm in A21 antibody precipitate |
|---|---|---|
| 0 | 339 | 284 |
| 1 | 624 | 624 |
| 3 | 1843 | 2069 |
| 6 | 2457 | 3405 |

Antibody-antigen precipitates were prepared as described in Materials and Methods. Approximately 50% of the counts extracted with Triton X-100 were subsequently precipitated by the antisera.

FIGURE 4.12

Analysis of Mitoribosomal Products of Strains D22 and A21 after 1 hour Derepression in Cycloheximide

The mitoribosomal products of strains D22 and A21 were labelled and SMP prepared as described in Materials and Methods. The products were analysed on 7.5% acrylamide gels. A and C show A21 products after 0 hours and 1 hour respectively in CHI; B and D show D22 products after 0 hours and 1 hour respectively in CHI. The final position of the tracking dye, BPB (▼) is marked.

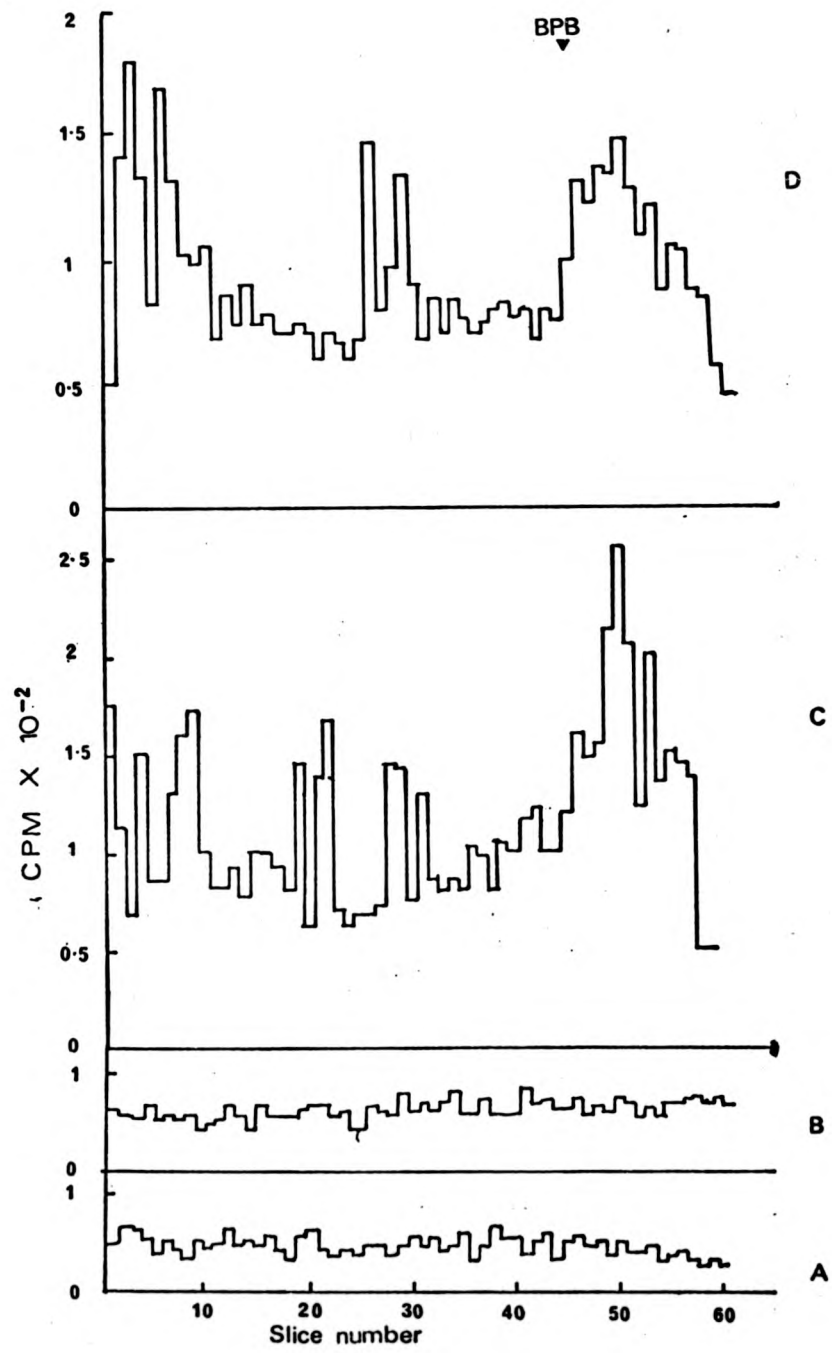

BPB
D
C
B
A
CPM X $10^{-2}$
Slice number
2
1·5
1
0·5
0
2·5
10
20
30
40
50
60

FIGURE 4.13

Analysis of Mitoribosomal Products of Strain D22 and A21 after 3 hours Derepression in Cycloheximide

The mitoribosomal products after 3 hours derepression were analysed as described in the legend to Fig. 4.12. A shows the distribution of $^{3}$H-leucine in D22 products and B in A21 products. The final position of the tracking dye BPB (▼) is marked.

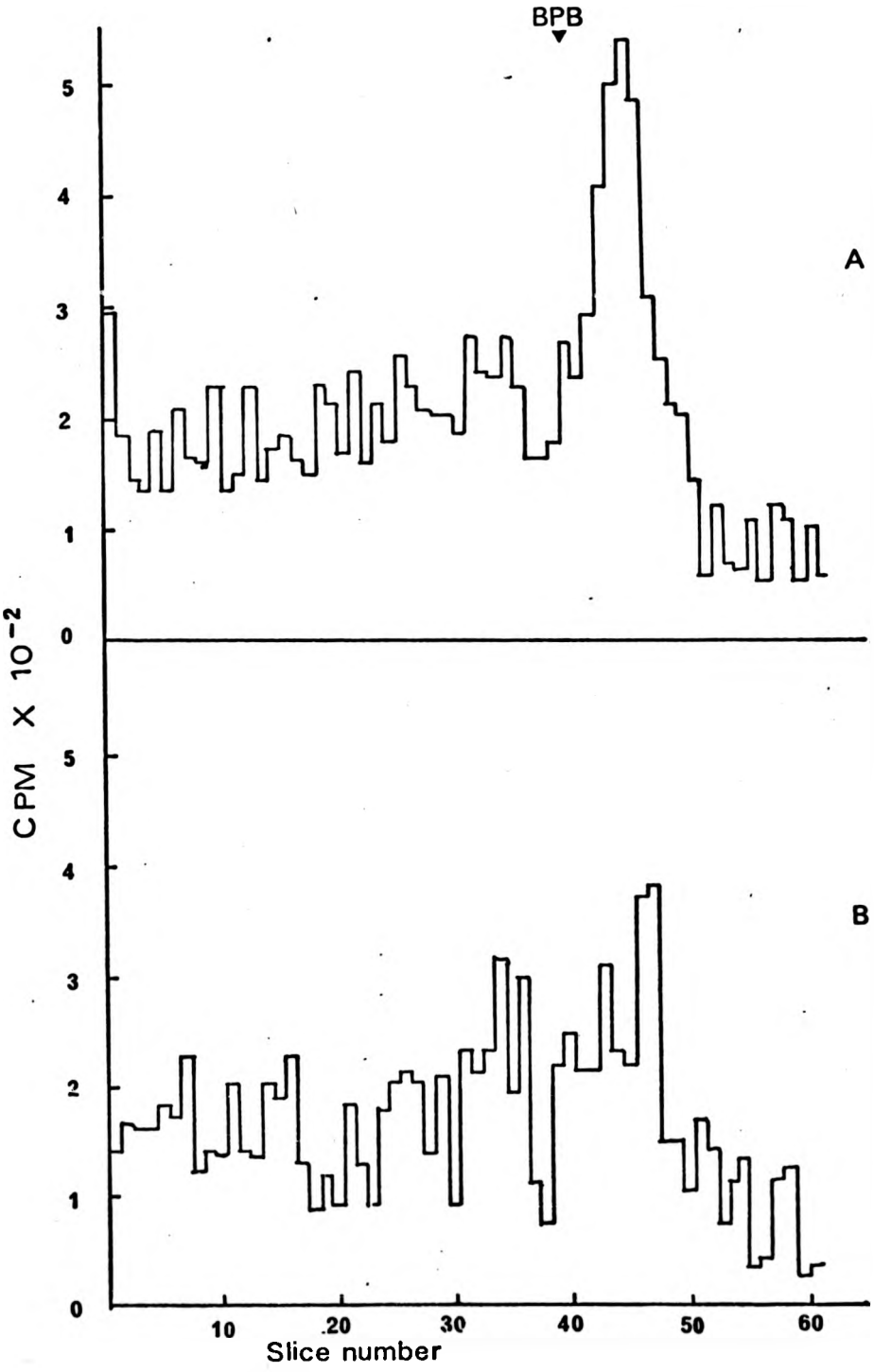
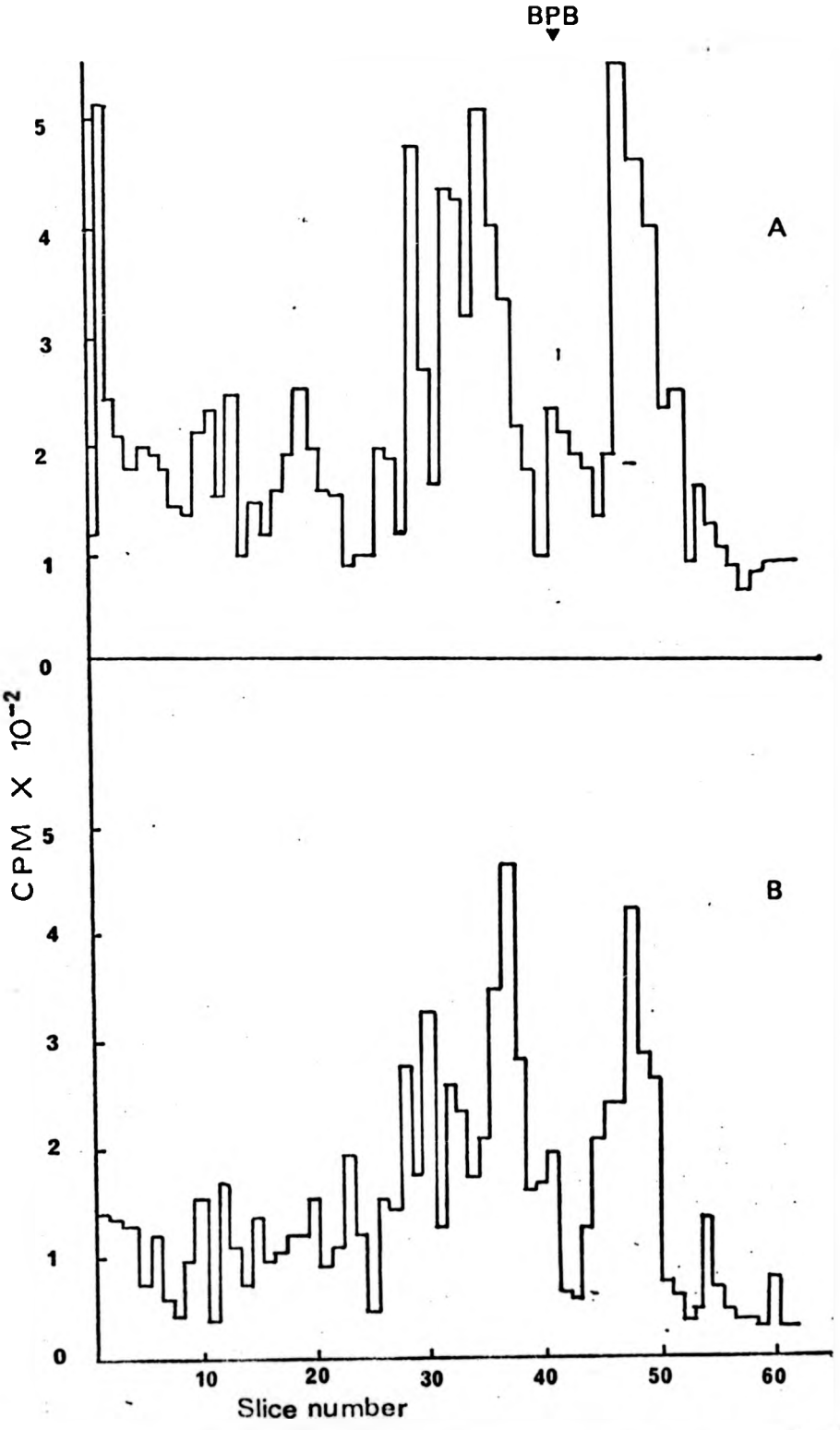

BPB
A
B
CPM X $10^{-2}$
0
1
2
3
4
5
10
20
30
40
50
60
Slice number

FIGURE 4.14

Analysis of Mitoribosomal Products of Strain D22 and A21 after 6 hours Derepression in Cycloheximide

The mitoribosomal products produced after 6 hours derepression in CHI were analysed as described in the legend to Fig. 4.12. A shows the distribution of $^{3}$H-leucine in D22 products and B in A21 products. The final position of the tracking dye BPB (▼) is marked.


BPB
A
B
CPM X $10^{-2}$
0
1
2
3
4
5
10
20
30
40
50
60
Slice number

that the electrophoresis patterns of SMP are not affected by treatment with alkali or organic solvent, again in contradiction of the findings of Tzagoloff. In order to see if the higher molecular weight peptide was the oligomeric form of the low molecular weight peptide as described by Tzagoloff et al. (1973) SMP were labelled, as described in Materials and Methods, for 17 hours and the products analysed as shown in Figs. 4.16a and 4.15a. The membranes were then homogenised in C:M, 2:1 v/v and the solvent dried off under a stream of warm air. The products were again analysed and the results are shown in Figs. 4.16b and 4.15b. In the case of both wild type and mutant the higher molecular weight peak has practically disappeared. It is difficult to say whether or not the higher molecular weight moiety has been converted into the lower form, as postulated by Tzagoloff (Tzagoloff and Akai, 1972) because considerable difficulty was encountered in solubilising the C:M treated SMP, with the result that a large proportion of the counts remain at the top of the gel. Antibody-antigen precipitates were made from wild type and mutant triton extracts as described in Materials and Methods, the two samples were mixed and analysed on polyacrylamide gels. The distribution of $^{3}$H-leucine is as shown in Fig. 4.17. The overwhelming majority of the label appears in the low molecular weight peptide with only trace amounts of the higher molecular weight species. The antibody-antigen complex had not been treated with alkali or organic solvent to bring about the transformation from high to low molecular weight peptide. The specificity of the antisera for OS ATPase is demonstrated in Fig. 4.18.

FIGURE 4.15

The Effect of Neutral Chloroform:Methanol on the $^{3}H$-Leucine Distribution in D22 Mitoribosomal Products

D22 SMP were labelled for 17 hours and analysed as described in Materials and Methods before (A) and after (B) treatment with neutral chloroform:methanol as described in Materials and Methods. The final position of the tracking dye BPB (▼) is marked.

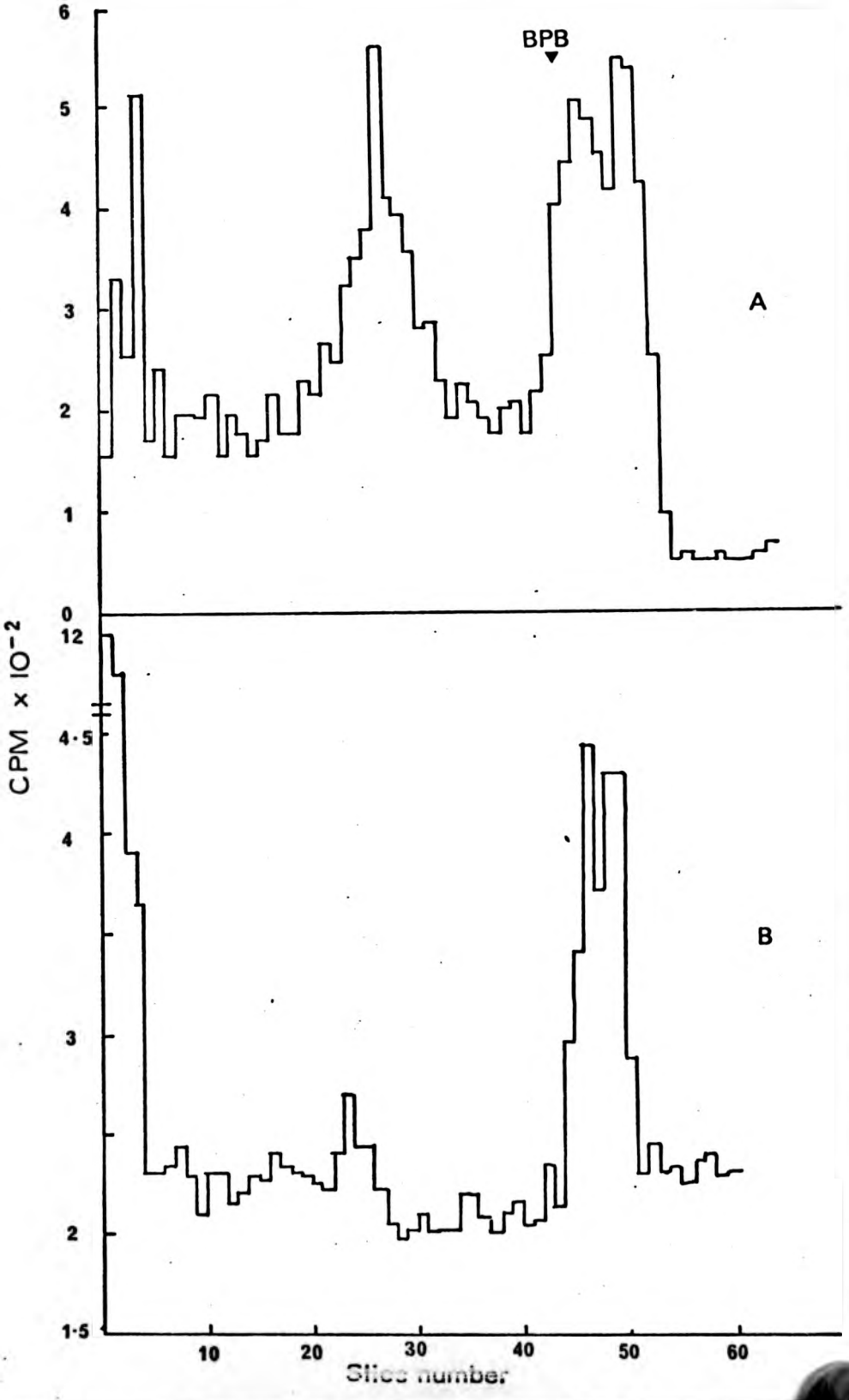

BPB
A
B
CPM x IO⁻²
6
5
4
3
2
1
0
12
4·5
4
3
2
1·5
10
20
30
40
50
60
Slice number

FIGURE 4.16

The Effect of Neutral Chloroform:Methanol on the Distribution of $^{3}$H-leucine in the Mitoribosomal Products of Strain A21

A21 SMP were labelled, prepared, and analysed before (A) and after (B) treatment with neutral chloroform:methanol as described in Materials and Methods. The final position of the tracking dye BPB (▼) is marked.

bution

21

re (A)

l as

n of

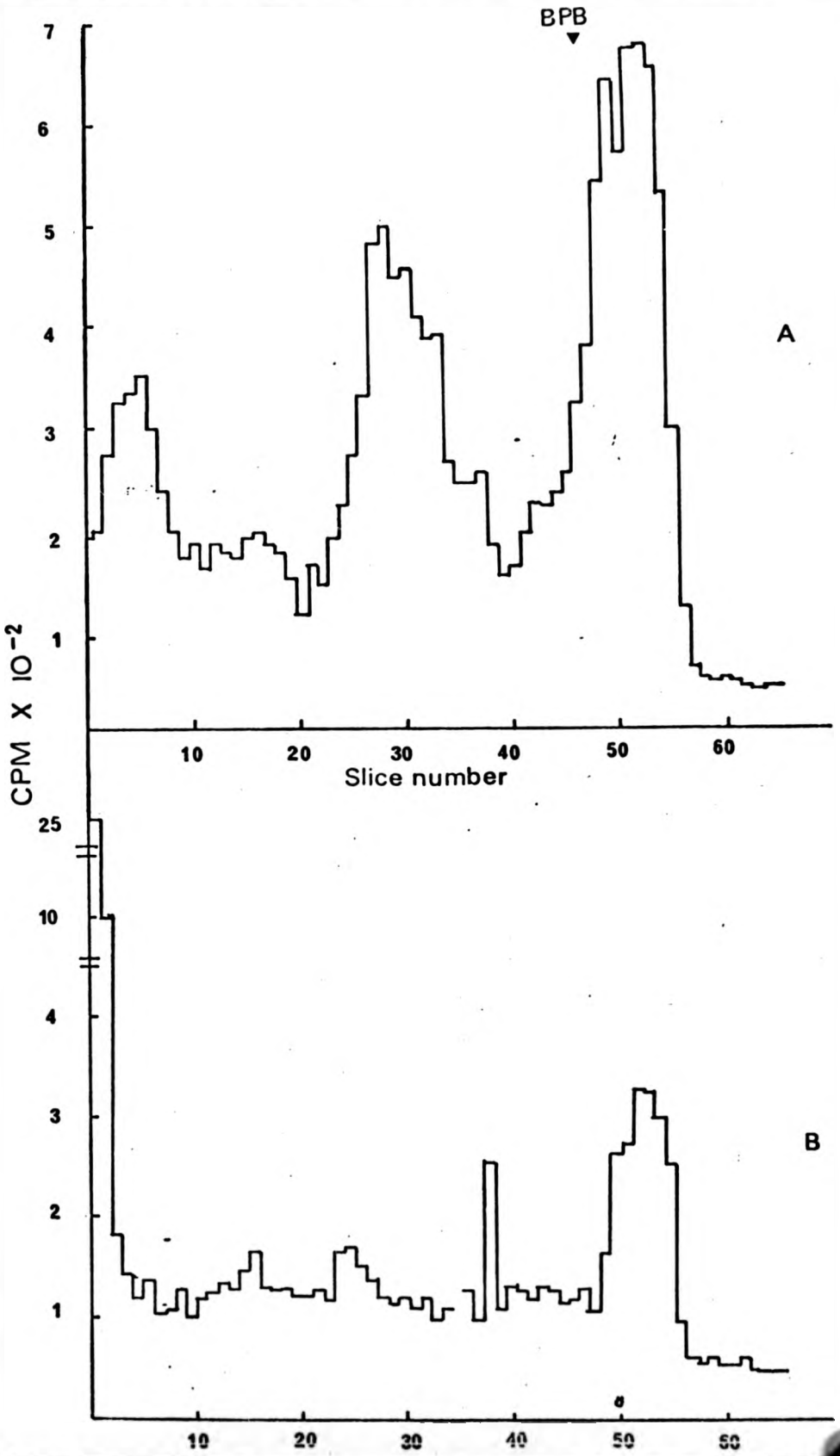
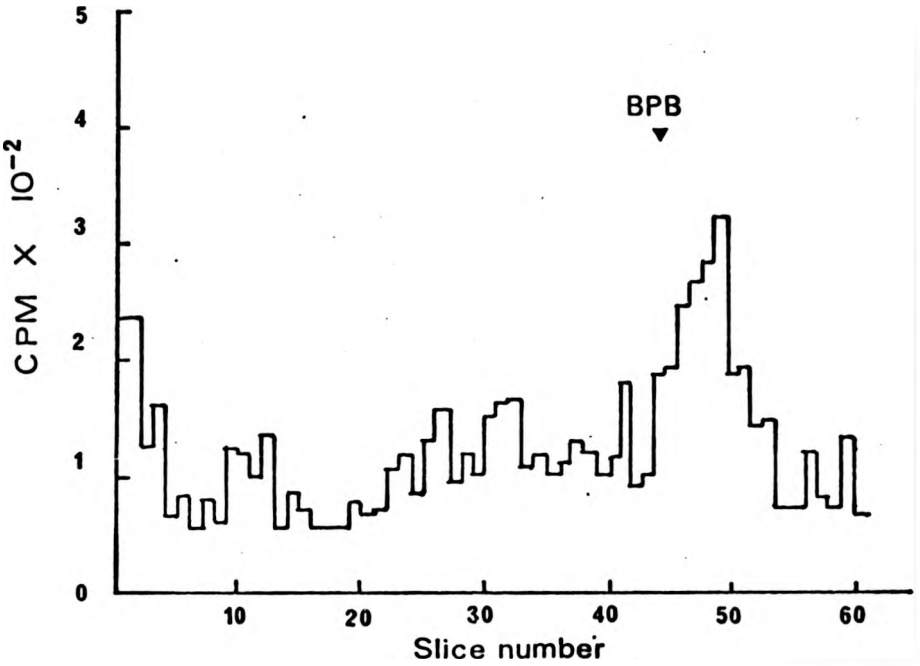


FIGURE 4.17

The Distribution of $^{3}$H-Leucine within Material Precipitated by Antisera to OS ATPase



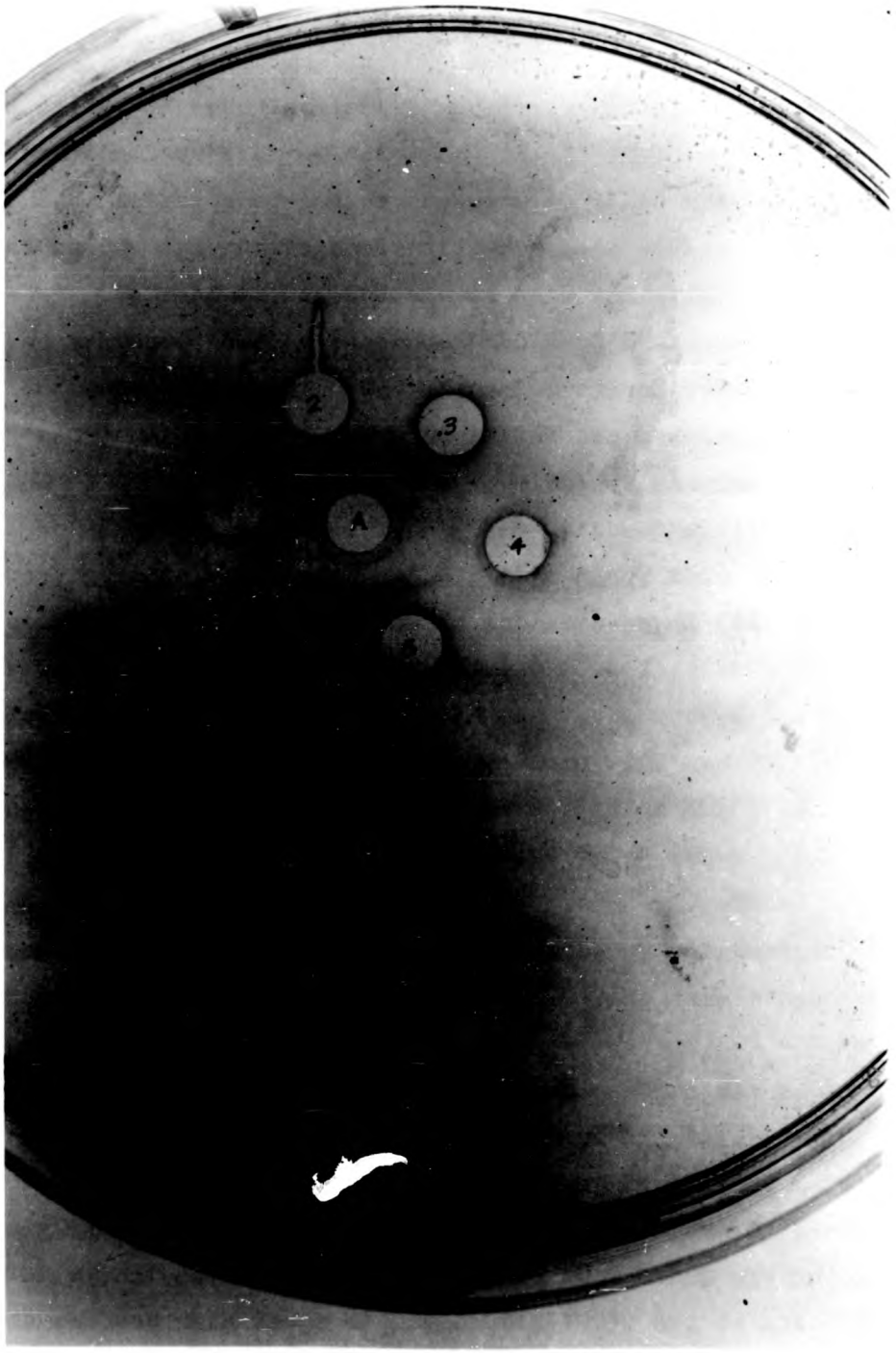

Precipitated A21 and D22 antibody-antigen complexes were prepared and sedimented as described in Materials and Methods. The precipitates were solubilised directly in 1% SDS, 1% mercaptoethanol, 10 mM $PO_4$ and 10% glycerol. The two solutions were combined and the total volume, 100 µl, applied to a 7.5% acrylamide gel. After electrophoresis the gel was sliced and counted. The final position of the tracking dye BPB (▼) is marked.

FIGURE 4.18

Specificity of Antisera raised against OS ATPase for OS ATPase

Well A contains 15 µl of antisera raised against OS ATPase.
Wells 1, 2 and 3 contain 30, 20 and 10 µgms respectively of D22 OS ATPase.
Well 4 contains 30 µgm of cytochrome oxidase.
Well 5 contains 20 µgm of A2 OS ATPase.

Well B contains 15 µl of preimmune sera with the same samples in the surrounding wells as Well A.


2
3
4

## 4.4 DISCUSSION

A series of cytoplasmically inherited mutants resistant to oligomycin have been selected. The kinetics of incorporation of $F_1$ ATPase produced on the cytoribosomes into OS ATPase located on the inner mitochondrial membrane have been studied. Changes in the reaction of the enzyme to a series of inhibitors has been monitored during incorporation into the membrane. The kinetics of production of mitoribosomal products necessary for incorporation of $F_1$ ATPase into OS ATPase has been monitored.

One of the mutants studied, A21, displays aberrant reactions to all the inhibitors tested immediately after the cells have undergone glucose repression and CAP poisoning, this effect disappears rapidly upon aeration in the absence of CAP. Within the limitations of the experimental techniques employed no significant changes in production of mitoribosomal products can be seen. An analogous mutant has been reported by Linnane and co-workers (private communication). The mutant L748 (TSO-R) is a temperature sensitive mutant which shows a reduced ATPase activity on growth at 19°C and only 40% of the mitochondrial ATPase activity is inhibitable by oligomycin. On transfer to media at 28°C containing CHI the enzyme becomes increasingly sensitive to oligomycin, in excess of 80% of the activity being inhibitable with oligomycin. This may be an organisational mutant or may display altered kinetics of production of a mitoribosomal peptide of the OS ATPase. Investigation into this aspect of the mutant are now in hand.

The mitoribosomal products found in this study are in broad agreement with those of Tzagoloff (1973) and do not agree with the findings of Ebner *et al.* (1973) that there is no low

low molecular weight mitoribosomal product. The system used by Ebner et al. is however different from that employed by Tzagoloff and that used in this study in that the cells are introduced directly into the labelling media containing CHI without preincubation with CAP. The studies of Tzagoloff (1971) indicate that in such a system the production of membrane factor necessary for the incorporation of $F_1$ ATPase will be negligible and that a prior incubation in CAP is necessary to build up a pool of cytoribosomal precursors before production of mitoribosomal products can be monitored in the absence of cytoribosomal activity. In conclusion we can say that the biogenesis of OS ATPase in the mutant A21 was aberrant but no firm conclusion could be reached concerning the cause of the aberrant behaviour.

# CHAPTER 5

## PURIFICATION AND CHARACTERISATION OF SUBUNIT 9 OF OS ATPase

### 5.1 INTRODUCTION

Proteolipids, as defined by Folch and Lees (1951) are protein-lipid complexes that are insoluble in water but soluble in organic solvents and occur generally in biological membranes (for review see Criddle and Willemott, 1969). Such a proteolipid moiety of molecular weight 7 to 10,000 daltons has been reported as the major product of the mitoribosomal system in the yeast Saccharomyces cerevisiae (Tzagoloff and Akai, 1972b; Murray and Linnane, 1972). The products may be different in Hela cells (Constantino and Attardi, 1975) and rat liver mitochondria (Burke and Beattie, 1973) although an analogous protein of unknown origin has been reported in ox heart mitochondria (Cattell et al., 1971; Steckhoven et al., 1972).

The work of Cattell and co-workers (1970; 1971) identified a low molecular weight proteolipid from ox heart mitochondria as the site of action of DCCD, a potent inhibitor of oxidative phosphorylation (see General Introduction), which covalently binds with a single proteolipid species and inhibits 95% of the reactions involving the synthesis or use of ATP. This inhibition takes place at a DCCD concentration range of 0.6 - 1.0 nmoles DCCD/mg mitochondrial protein, which is similar to the concentration range of the cytochrome components of the electron transport chain in ox heart mitochondria and hence the concentration at which intermediates in ATP synthesis would be expected to exist (Beechey et al., 1967).

The work of Tzagoloff and co-workers (Tzagoloff and Akai, 1972b; Tzagoloff et al., 1973) indicates that the major mitoribosomal product is a single low molecular weight proteolipid and they have elegantly demonstrated that this peptide appears identical with sub unit 9 of the OS ATPase. There is however considerable controversy over this issue as Schatz's laboratory have been unable to demonstrate the presence of such a lipid (Ebner et al., 1973) but this may be due to the deficiencies in their system referred to in the Discussion of Chapter 4.

The inhibitors DCCD and oligomycin have very similar functional effects (Roberton et al., 1968). In view of this and the identification of a low molecular weight proteolipid as sub unit 9 of OS ATPase and DCCD binding protein it was thought that a mutation affecting oligomycin sensitivity may involve a change in the protein moiety responsible for DCCD binding. Hence subunit 9, a mitoribosomal product, presumably coded for by mitochondrial DNA is perhaps the peptide responsible for oligomycin sensitivity/resistance and may be altered in mutations selected for oligomycin resistance.

Tzagoloff (Tzagoloff and Akai, 1972b; Tzagoloff et al., 1973) reported that extraction of SMP with neutral chloroform: methanol, 2:1 v/v, results in practically exclusive extraction of sub unit 9 of ATPase. This extract has been subjected to sequential ether washes, subjected to T.L.C. followed by further ether washes. The resulting proteolipid was judged homogenous on the criteria of gel electrophoresis (Figs. 5.2, 5.3).

Having isolated and characterised subunit 9 of the OS ATPase it was intended to obtain peptide maps of sub unit 9

from the series of mutants used throughout this study and thus detect any peptide alteration giving rise to lowered sensitivity to oligomycin.

## 5.2 METHODS AND MATERIALS

### Materials

All organic solvents were redistilled from analar grade.

Siclica gel 60 plates, 20 cm x 20 cm x 2 mm, were produced by Merck and obtained through Anderman and Co., Ltd., London.

### Growth of Cells

The conditions for the growth of cells were as described in Chapter 3.

### Preparation of SMP

SMP were prepared by Braun shaker as described in Chapter 2.

### Labelling of Mitoribosomal Products

Mitoribosomal products were labelled as described in Chapter 4 except that the cells were grown for 17 hours in the presence of $^{3}$H-leucine and CHI.

### Protein Estimation

Protein was estimated by the procedure of Lowry et al., (1951). Where proteolipid estimations were carried out the solvents were first evaporated off and the residues dissolved by boiling in 1% SDS for 2 minutes.

### Purification of Low Molecular Weight Proteolipid

SMP were brought to a concentration of 20 mg/ml by the

addition of 10 mM Tris acetate pH 7.5. 10 volumes of analar acetone were added and the mixture stirred for 1 hour at room temperature. The suspension was spun for 10 minutes at 2000 r.p.m. in glass bottles in a Sorvall G.S.A. rotor. The precipitate was resuspended in 5 volumes of 90% acetone and stirred at room temperature for 20 minutes, the suspension was then spun again at 2000 r.p.m. for 10 minutes in glass bottles in a Sorvall G.S.A. rotor.

The pellet was homogenised in three volumes of chloroform: methanol 2:1 v/v and allowed to stand at 55°C for 30 minutes. The suspension was spun for 10 min at 16,000 r.p.m. in stainless steel tubes in a Sorvall SS34 rotor. The supernatant was carefully decanted, the pellet being very easily disturbed and any contamination after this point being difficult to remove, and dried down under vacuum. The residue was redissolved in C:M 2:1 v/v (usually about half the original volume used to extract the SMP) and precipitated overnight at 4°C with 4 volumes of ether. The precipitate was removed by spinning at 3,000 r.p.m. for 10 min in glass tubes in a Sorvall SS34 rotor and redissolved in C:M 2:1 v/v. The ether precipitation step was repeated two more times and the final ether precipitate taken up in as small amount as possible of C:M 2:1. The sample was subjected to T.L.C. being applied in a streak on a Merck Silica Gel 60 20 cm x 20 cm x 2 mm plate with a small amount of radiolabelled proteolipid at one end of the streak to act as a protein marker. The plate was developed in a solvent system consisting of 130 ml $CHCl_3$: 50 ml $CH_3OH$: 7.4 ml $H_2O$; 0.62 ml 6N HCl and developed in a tank saturated with iodine vapour. A typical T.L.C. pattern is

shown in Fig. 5.1. The major bands, labelled 1 and 2 and their equivalent region of gel above the radiolabelled spot were removed from the plate. The removed gel was thoroughly ground with a small glass rod and just enough water, about 2 drops, added to the gel to dampen it. A small volume of C:M 2:1, 20 mM HCl, about 2 ml, was added to the gel and then a further 10 µl 6N HCl was added. The suspension was thoroughly mixed and allowed to stand for 30 min at room temperature and was occasionally stirred. The silica gel was spun off and re-extracted as before with the ommision of the 10 µl of 6N HCl. The supernatants were pooled and dried down by heating to 50°C and passing air over the surface of the solution. The residue was taken up in a small volume of C:M 2:1, about 1 ml, and subsequently precipitated with the addition of 4 volumes of cold ether, this procedure was repeated and the final residue taken up in a small volume of C:M 2:1. The recoveries of protein and radiolabel are shown in Tables 5.1 and 5.2.

Disc Gel Electrophoresis

20 µg of $F_9$ recovered from the T.L.C. plate was dried down and the residue redissolved in 50 µl of 2% SDS, 2% mercaptoethanol, 10% glycerol and 10 mM $PO_4$ by boiling for 1 minute and subsequently heating at 70°C for 20 min. 3 µl of a 0.005% aqueous solution of BPB was added and the solution applied to the top of a 7.5% acrylamide gel. The gel was subjected to a current of 8 milliamps for 4 hours. The conditions described for electrophoresis were those described by Weber and Osborn (1969). The gels were stained in 0.25%

FIGURE 5.1

Thin Layer Chromatography of Neutral Chloroform:Methanol Extract of Yeast Sub Mitochondrial Particles

The preparation of extract and conditions of chromatography were as described in Materials and Methods. The solvent front was allowed to progress to the top of the plate. Bands 1 and 2 were removed for further investigation.

s.

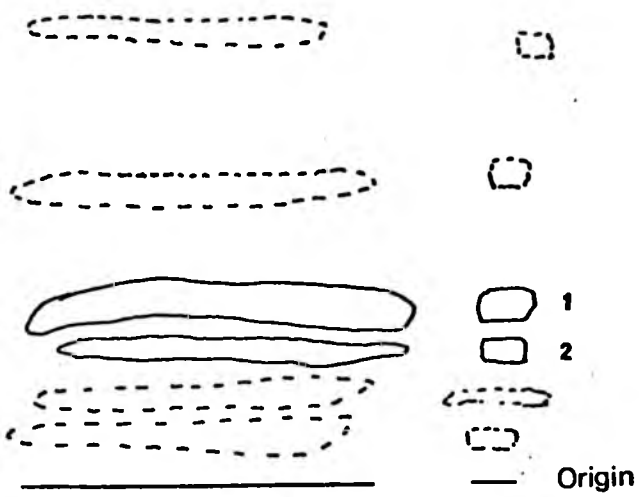


TABLE 5.1

Recoveries of Protein During Sub unit 9 Purification

| | |
|---|---|
| Cells ( gm wet weight) | 183 |
| SMP (mg protein) | 940 |
| Purified $F_9$ (mg Protein) | 2 |

TABLE 5.2

Recovery of Counts During Proteolipid Purification

| Fraction | Total c.p.m. |
|---|---|
| SMP | 168,000 |
| C:M extract | 63,200 |
| Band 1) recovered from T.L.C. plate | 56.600 |
| Band 2) recovered from T.L.C. plate | 160 |
| Counts left at origin of T.L.C. plate | 748 |

100 µl of D22 SMP (79,000 c.p.m./mg) and 50 µl A21 SMP (90,000 c.p.m./mg) labelled as described in Materials and Methods and containing a total of 168,000 c.p.m. $F_9$ was purified as laid out in Materials and Methods.

solution of amido black in methanol : acetic acid : water 5 : 1 : 5 v/v. Staining with coomassie brilliant blue gave very faint staining.

An aliquot of radiolabelled $F_9$ recovered from the T.L.C. plate was also subjected to polyacrylamide gel electrophoresis. This gel was sliced and counted as described in Materials and Methods, Chapter 4.

Peptide Mapping

A host of problems were encountered in attempting to render the protein amenable to the production of peptide maps and in attempting to obtain reproducible peptide maps. The most intractible problem was the insolubility of the peptide in all but the harshest solvents such as SDS, formic acid and pyridine. The first approach was to attempt to solubilise the protein in SDS and maintain the detergent at low enough concentration to allow the protease to remain active. The general procedure employed was as follows: 0.5 ml of C:M 2:1 containing 400 - 500 µg of protein was dried down over an air jet, 0.2 ml of 1% SDS was added and the pH adjusted to 7.5 with the addition of NaOH, the solution was boiled for 1 min and heated at 70°C for 20 min. Even after such harsh treatment the residue was not completely dissolved and the undissolved residue was removed by centrifugation. The sample was diluted to 2 ml and 0.2 ml removed for a predigested control. 10 µg of trypsin was added to the remainder. It was difficult to say whether digestion was proceeding by following the pH but peptide analysis by T.L.C. and high voltage electrophoresis indicated very incomplete digestion and variable results.

Attempts to render the protein more soluble by substituting the amino groups of the peptide by maleylation and succinylation and increase the negative charge on the protein. The procedures adopted were those laid out by Sund (1971). Neither procedure resulted in a more soluble peptide and as subsequent partial digestion by trypsin was not affected it was thought that the substitution reaction had not taken place.

Amino Acid Analysis

Approximately 80 μgm of $F_9$ dissolved in C:M 2:1 was pipetted in a hydrolysis tube and taken to dryness. 1 ml of 6N HCl was added and the tube evacuated and sealed and placed in an oven at 110°C for 24 hours. The tube was opened and the HCl removed under vacuum at 50°C. The residue was dissolved in 1 ml $H_2O$ and subjected to analysis on a Beckman 120C amino acid analyser.

## 5.3 RESULTS

This Chapter describes the purification and characterization of a low molecular weight proteolipid identified by Tzagoloff et al. (1973) as subunit 9 of the OS ATPase. The protein is electrophoretically pure (Fig. 5.2 and 5.3). The amino acid analysis of the protein displays extreme hydrophobicity (Table 5.3), the distribution of polar and apolar amino acid residues shows good agreement with that reported by Tzagoloff et al. (1973). The differences in serine and methionine content may be due to strain differences; histidine, proline and methionine are present as traces in insufficient amounts to account for 1 residue of a 7,800 daltons molecular weightpeptide (Tzagoloff, 1973) and probably indicate a low level of

FIGURE 5.2

Disc Gel Electrophoresis of Sub unit 9 of the OS ATPase

The sample of sub unit 9 for electrophoresis was prepared as described in Materials and Methods. The sample was electrophoresed at 8 milliamps/gel for approximately 4 hours down a 7.5% acrylamide gel. The gel was stained with amido black and destained. The final position of the tracking dye, BPB (▼) is shown.

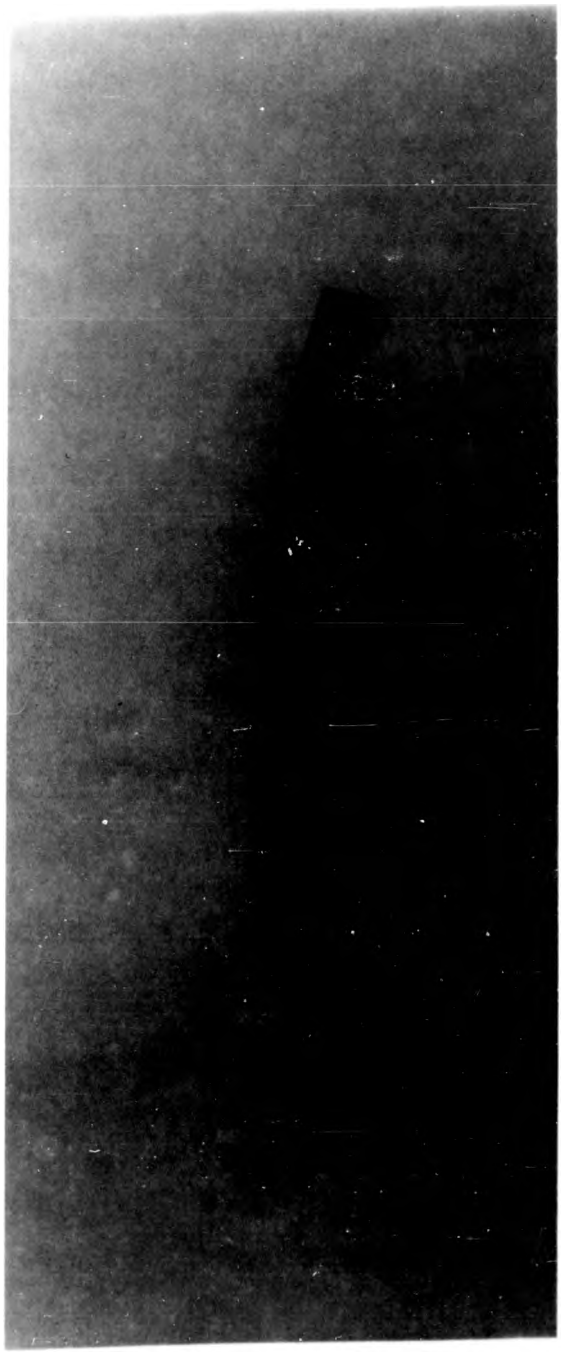
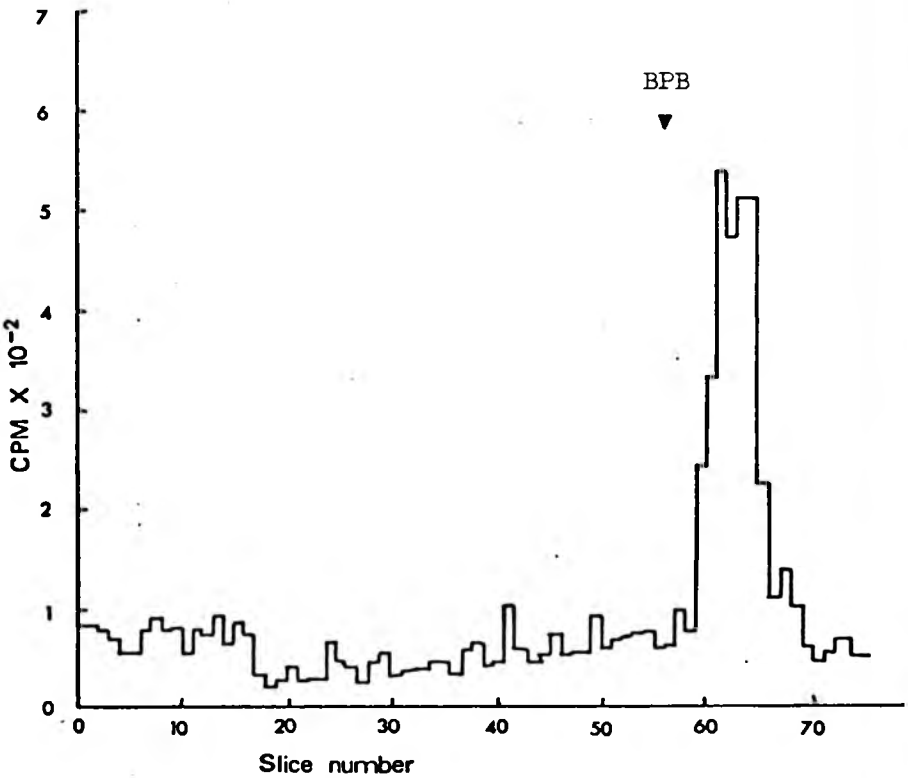


FIGURE 5.3




Disc Gel Electrophoresis of $^{3}$H-Leucine Labelled Sub unit 9

A sample of tritiated sub unit 9 was purified by T.L.C. and ether washing. The sample was dried down and solubilised directly in 1% SDS, 1% mercaptoethanol, 10 mM $PO_4$ and 10% glycerol and applied to the top of a 7.5% acrylamide gel. After electrophoresis the gel was sliced and counted following the procedures laid out in Materials and Methods, Chapter 4.

TABLE 5.3

Amino Acid Composition of Sub unit 9

| Amino Acid | Mole per cent amino acid in $F_9$. |
|---|---|
| Lysine | 5.20 |
| Histidine | 1.39 |
| Arginine | 3.08 |
| Aspartic Acid | 4.83 |
| Threonine | 3.57 |
| Serine | 15.54 |
| Glutamate | 7.73 |
| Proline | trace |
| Glycine | 14.95 |
| Alanine | 10.01 |
| Valine | 8.16 |
| Methionine | 1.4 |
| Isoleucine | 5.33 |
| Leucine | 8.37 |
| Tyrosine | 1.40 |
| Phenylalanine | 8.01 |
| Total mole per cent | 98.97 |

The values represent the average of two analyses.

contaminant.

The peptide proved to be highly insoluble in any solvent system rendering it amenable to protease activity. Attempts to render the peptide more soluble were unsuccessful. Attempts to covalently bind $^{14}$C-DCCD to the proteolipid; in a fashion analogous to Cattell et al. (1971) both at the level of SMP and purified OS ATPase were unsuccessful, the $^{14}$C-DCCD being easily removed by washing. The DCCD still displayed an inhibitory effect but failed to bind, this has been the experience of other workers attempting to bind $^{14}$C-DCCD to yeast SMP (M. Partis, Personal Communication; Broughall, 1972).

## 5.4 DISCUSSION

The proteolipid, the purification and characterisation of which is described in this Chapter, has been identified by Tzagoloff and co-workers as sub unit 9 of the OS ATPase. Unlike the low molecular weight proteolipid from ox heart mitochondria described by Cattell et al. (1971) it does not covalently bind DCCD. The proteolipid has been purified from wild type and oligomycin resistance mutants A21, A19 and A15 with a view to the possible identification of an altered peptide using well established methods of peptide mapping. The proteolipid from the mutants showed altered properties during purification from that of the wild type. The variability and absolute value of yields of wild type and mutant proteolipid were similar. The thin layer chromatography pattern obtained during purification were practically identical, no significant changes in the Rf values of the proteolipid being detectable. On analysis by disc gel electrophoresis all the mutants gave

the same banding pattern as that obtained with wild type proteolipid i.e. that shown in Fig. 5.2. Thus it was concluded that the mutant mitochondrial membranes contained similar quantities of sub unit 9 to wild type membranes. From the chromatography and electrophoretic data it was concluded that on the basis of these two criteria that none of the sub unit 9 purified from the mutants was grossly altered in size or composition. This was a somewhat expected result as sub unit 9 is the major mitoribosomal product associated with the OS ATPase and in view of its distinct hydrophobicity it is thought to play an important role in the structural integrity of the enzyme. In Chapter 3 it was shown that the enzyme from the mutants was extractable in an integrated state, if sub unit 9 had been absent or greatly depleted it was felt that the integrity of the mutant enzyme would have suffered and an altered yield of enzyme of structural integrity would have resulted. Thus it was thought that any mutation causing inhibitor resistance would be of a very limited nature and so it was proposed to further analyse the proteolipid from wild type and mutant strains by peptide analysis.

Insurmountable problems were encountered in attempting to carry out these analyses. The problem of peptide solubility in any solvent system amenable to protease activity was found to be an intractable one, no reproducible results or maps showing total digestion being obtained. The problems outlined above met with in the purification and characterisation of mitochondrial proteolipids have been encountered by other workers in the field. The publication of a tentative identification of a low molecular weight mitoribosomal proteolipid

by Murray and Linnane in 1972 (Murray and Linnane, 1972) which promised further characterisation of the proteolipid has not been followed by further publications on the subject and so presumably these workers are also encountering solubility problems. Further work by Tzagoloff (A. Tzagoloff, Private Communication) on peptide mapping of the proteolipid has been inconclusive. Attempts by workers in Beechey's laboratory have met with intractable solubility difficulties in attempting to produce peptide maps of $^{14}$C-DCCD labelled proteolipid from ox heart mitochondria (P.R. Avner, Private communication). In view of the extreme hydrophobicity and insolubility of the proteolipid in any physiological solvent system amenable to protease activity it is thought that any further analysis will require the development of novel techniques of peptide analysis.

Some of the work described in this Chapter was carried out in the laboratory of Dr. A. Tzagoloff during June and July, 1972.

## GENERAL DISCUSSION

The factor(s) responsible for the changes in inhibitor sensitivity of those mutants cited in the Introduction were shown to reside in the membrane fraction of the OS ATPase. The OS ATPase was shown to possess, in addition to a site of action of oligomycin, a site of action of venturicidin and of TET. Problems arose during the inhibition studies on the solubilised enzyme because of the extreme susceptibility of apparent inhibitor sensitivity of the enzyme to levels of both detergent and phospholipid in the assay medium. Because of these factors it would prove difficult to arrive at a true value of the amount of inhibitor required to inhibit the enzyme activity by 50%, i.e. an $I_{50}$ value, for the wild type enzyme but as this study was essentially a comparative one using several different inhibitors to test for enzyme integrity and rigid control was exercised over the detergent and phospholipid levels in the assay medium it is felt that the differentials in sensitivity found between wild type and mutants were true ones. The work with isonuclear constructed diploids shows an in vitro effect of inclusion of a $TET^R$ mutation, until this time cells displaying a resistance to TET at the whole cell level have shown no aberrant behaviour at a mitochondrial, SMP or solubilised enzyme level. It is felt that the results presented in Chapter 4 concerning the aberrant kinetics of OS ATPase formation in mutant A21 could not be improved upon without a heavy investment in better peptide analytical apparatus and radiolabelled amino acids. The work with sub unit 9 of the OS ATPase described in Chapter 5 would seem to

present the most intractable problems to be faced yet and would seem to be the most promising avenue for some rapid progress in the investigation of this problem.

Several promising approaches suggest themselves, the most direct approach to the analysis of this peptide would seem to be a complete sequencing of peptide prepared from wild type and mutant cells. An analysis of a peptide composed of some 50 amino acid residues could be undertaken using an amino acid sequencer. The major advantage of this approach is the use of a pyridine based solvent system which would ensure solubility of the proteolipid. To obtain sufficient material for such an analysis it would be beneficial to allow the cells to grow in the presence of CAP and then allow the cells to derepress in the presence of CHI as described by Tzagoloff (1971). This treatment results in an overproduction of sub unit 9 giving a three fold increase in the amount of sub unit 9. A second approach which may prove fruitful would be to modify the method by which the peptide maps are visualised. When visualising peptide maps with peptide specific stains the amount of protease used must be rigidly controlled to prevent contamination of the map by large amounts of protease self digestion products. To overcome the problem of being strictly limited in the amount of protease it is possible to use, it should be possible to produce $^{14}C$-labelled sub unit 9 of high specific activity, Autoradiography could now be used to visualise the proteolytic products of such a labelled peptide. Two advantages would be gained by such an approach: 1) Only those peptides resulting from digestion of radio-labelled sub unit 9 would be visualised and hence the protease

can be used in very high concentrations and should result in complete digestion of the proteolipid. 2) Autoradiographic techniques for detecting even low energy radioactive emitters are becoming increasingly sensitive with the advent of such novel innovations as scintillant coated film. With this increased sensitivity it should be possible to visualise much smaller amounts of digested peptide than is possible with more traditional methods such as ninhydrin staining.

The analysis of sub unit 9 from wild type and mutant cells still leaves the problem of purification and analysis of the other three mitoribosomal products associated with the OS ATPase, any of which may be carrying the expressed mutation. These peptides are present in very small amounts in the OS ATPase relative to the larger cytoribosomally produced sub units and therefore their purification becomes a more difficult task. Attempts were made to purify these sub units on the apparatus described in the Appendix to this Thesis by separating the sub units of OS ATPase on the preparative gel. In the light of this experience a potentially more profitable approach would have been to pass the enzyme through a procedure to enrich the relative amounts of the mitoribosomal products. Two possible procedures are suggested here: 1) When SMP are extracted with acidic chloroform:methanol 2:1 v/v, 10 mM HCl, the resulting extract contains at least eight proteins seperable on polyacrylamide gels. However if the purified OS ATPase is extracted with acidic chloroform:methanol only three or four bands appear on polyacrylamide analysis of the extract and three have been tentatively identified as mitoribosomal in origin (A. Tzagoloff, Private communication).

2) If the OS ATPase is dissociated in the presence of urea and guanidine hydrochloride and the solution dialysed against two changes of water for 24 hours the two highest molecular weight components of the enzyme, both cytoribosomal in origin, precipitate out of solution (A. Tzagoloff, Private communication). Thus both these procedures result in a greatly enriched mitoribosomally produced fraction which is much more amenable to separation and purification by gel electrophoresis than the whole enzyme.

The problem throughout much of this work has been to identify the aberrant peptide(s) responsible for the phenomena of inhibitor resistance(s). The work so far undertaken indicates strongly that the change in the peptide is a subtle one and does not involve large or complete deletions of sub units or gross structural changes within the enzyme. Given such a subtle change and the difficulties encountered in attempting to analyse mitoribosomal products outlined in Chapter 5, an early solution to such a problem will not be easily found. Perhaps a more fruitful approach would be to select mutations which have a more drastic effect than increased inhibitor resistance, such mutations may well result in a much larger structural and therefore more easily identifiable change in the enzyme. Just such an approach has recently been used by Tzagoloff and Needleman (1975) in studies on cytochrome oxidase. Briefly the procedure involved screening for mutants without oxidative phosphorylation capability but displaying functional mitoribosomal apparatus. Further screening of the mutants involved enzyme purification and polyacrylamide gel analysis to look for aberrant or absent mitoribosomal products.

Such an approach could be used in an investigation of and possible assigning of OS ATPase peptide roles in oxidative phosphorylation.

## REFERENCES


Aldridge, W.N. and Cremer, J.E. (1955) Biochem. J. 61, 406.

Aldridge, W.N. (1957) Biochem. J. 67, 423.

Aldridge, W.N. (1958) Biochem. J. 69, 367.

Aldridge, W.N. and Threlfall, C.J. (1961) Biochem. J. 79, 214.

Aldridge, W.N. and Street, B.W. (1964) Biochem. J. 91, 287.

Aldridge, W.N. and Street, B.W. (1970) Biochem. J. 118, 171.

Aldridge, W.N. and Street, B.W. (1971) Biochem. J. 124, 222.

Aloni, F. and Attardi, G. (1971) J. Mol. Biol. 55, 251.

Avner, P. and Griffiths, D.E. (1970 ) FEBS Lett. 10, 202.

Avner, P. and Griffiths, D.E. (1973a) Eur. J. Biochem. 32, 301-311.

Avner, P. and Griffiths, D.E. (1973b) Eur. J. Biochem. 32, 312-321.

Avron, M. and Shavit, N. (1965) Biochim. Biophys. Acta 109, 317.

Beechey, R.B., Holloway, C.T., Knight, I.G. and Roberton, A.M. (1966) Biochem. Biophys. Res. Commun. 23, 75.

Beechey, R.B., Roberton, A.M., Holloway, C.T. and Knight, I.G. (1967) Biochem. A.C.S. 12, 3867.

Bragg, P.D. and Hou, C. (1972) FEBS Lett. 28, 309.

Brooks, J.C. and Senior, A.E. (1971) Arch. Biochem. Biophys. 147, 467.

Broughall, J.M. (1973) Ph. D. Thesis, University of Warwick.

Brufani, M., Keller-Schierlein, W., Loeffler, W., Mansperger, I. and Zahner, H. (1968) Helv. Chim. Acta 51, 1297.

Brufani, M., Cellai, L., Musca, C., and Keller-Schierlein, W. (1972) Helv. Chim. Acta 55, 2329-2346.

Bulos, B. and Racker, E. (1968) J. Biol. Chem. 243, 3901.

Burke, J.P. and Beattie, D.S. (1973) Biochem. Biophys. Res. Commun. 51, 349-356.

Capaldi, R.A. (1973) Biochem. Biophys. Res. Commun. 53, 1331.

Cattell, K.J., Knight, I.G., Lindop, C.R. and Beechey, R.B. (1970) Biochem. J. 117, 1011-1013.

Cattell, K.J., Knight, I.G., Lindop, C.R. and Beechey, R.B. (1971) Biochem. J. 125, 169-177.

Catterall, W.A. and Pedersen, P.L. (1971) J. Biol. Chem. 246, 4987.

Coen, D., Deutsch, J., Nett er, P, Petrochilo, E. and Slonimski, P.P. (1970) Symp. Soc. Expt. Biol. ed. Miller, P. 24, "The Development and Relationship of Cell Organelles" 449. Cambridge University Press.

Cogan, U., Shinitzky, M., Weber, G. and Nishida, T. (1973) Biochem. A.C.S. 12, 521.

Constantino, P. and Attardi, G. (1975) J. Mol. Biol. 96, 291-306.

Cosson, J. and Spiridakis, A. (1974) Biochem. Biophys. Res. Commun. 59, 1039.

Cox, G. and Gibson, F. (1974) Biochim. Biophys. Acta 346, 1-25.

Criddle, R. and Willemott, J. (1969) in "Structural and Functional Aspects of Lipoprotein in Living Systems" Eds. Tvia, E. and Scanu, A. p. 173 Academic Press.

Danielson, L. and Ernster, L. (1963) Biochem. Biophys. Res. Commun. 10, 91.

Ebner, E., Mennucci, L. and Schatz, G. (1973) J. Biol. Chem. 248, 5360-5368.

England, J.M. and Attardi, G. (1974) J. Mol. Biol. 85, 433-444.

Ephrussi, B., Hottinguer, H. and Roman, H. (1955) Proc. Nat.

Acad. Sci. U.S. 41, 1065.
Ephrussi, B. Hottinguer, H. Roman H. (1955) Proc Nat. Acad. Sci. U.S. 41, 1065.
Ephrussi, B., Jacob, H. and Grandchamp, S. (1966) Genetics 54, 1.

Farron, F. (1970) Biochem. 9, 3823.

Folch, J. and Lees, M. (1951) J. Biol. Chem. 191, 807.

Gibson, F. (1971) Search, 2, 425-429.

Glehn, M. von., Norrestans, R., Kierkegaard, P., Maron, L. and Ernster, L. (1972) FEBS Lett. 20, 267

Green, A.P., Sweetman, A.J., Hooper, M., Cattell, K.J., Lindop, C.R. and Beechey, R.B. (1973) Biochem. Soc. Trans. 1, 410-413.

Griffiths, D.E., Avner, P.R., Lancashire, W.E. and Turner, J.R. (1972) Int. Symp. Biochem. Biophys. of Mito. Membranes. Eds. Azzone, G.F., Carafoli, E., Siliprandi, N. and Quagliariello, E. Academic Press, London.

Griffiths, D.E. and Houghton, R.L. (1974) Eur. J. Biochem. 46, 157-167.

Griffiths, D.E., Houghton, R.L. and Lancashire, W.E. (1975a) Eur. J. Biochem. 51, 393-402.

Griffiths, D.E., Lancashire, W.E. and Meadows, P. (1975b) Eur. J. Biochem. 51, 403-413.

Harold, F.M., Baarda, J.R., Baron, C. and Abrams, A. (1969) J. Biol. Chem. 244, 2261.

Hatase, O., Wakabayashi, T., Allman, D.W., Southard, J.H. and Green, D.E. (1973) Bioenergetics 13, 1-15.

Horstman, L.L. and Racker, E. (1970) J. Biol. Chem. 245, 1336.

Houghton, R.L., Lancashire, W.E. and Griffiths, D.E. (1974) Biochem. Soc. Trans. 2, 210-213.

Howell, S.H. and Moudrianaris, E.N. (1967) Proc. Nat. Acad. Sci. U.S. 58, 1261.

Hsia, J., Long, R.,Hrúsra, F. and Gesser, H. (1972) Biochim. Biophys. Acta 290, 22-31.

Hubbell, W. and McConnell, H. (1971) J. Am. Chem. Soc. 93, 314.

Huijing, F. and Slater, E.C. (1961) J. Biochem. (Japan) 49, 493.

Jost, P., Libertini, L., Herbert, V. and Griffiths, O.H. (1971) J. Mol. Biol. 59, 77-98.

King, E.J. (1932) Biochem. J. 26, 293.

Knight, I.G., Holloway, C.T., Roberton, A.M. and Beechey, R.B. (1968) Biochem. J. 109, 27.

Knowles, A.F. and Penefsky, H.S. (1972a) J. Biol. Chem. 247, 6624.

Knowles, A.F. and Penefsky, H.S. (1972b) J. Biol. Chem. 247, 6617.

Lamb, A.J. (1968) Biochim. Biophys. Acta 161, 415.

Lancashire, W.E. and Griffiths, D.E. (1975) Eur. J. Biochem. 51, 377-392.

Langcake, P., Beechey, R.B., Lindop, C.R., Wickins, S.G.A., Leworthy, D.P., Wiggins, D.E. and Broughall, J.M. (1974) Biochem. Soc, Trans. 2, 202-205.

Lardy, H.A., Connelly, J. and Johnson, D. (1964) Biochem. A.C.S. 3, 1961-1968.

Lardy, H.A., Witonsky, P. and Johnson, D. (1965) Biochem. A.C.S. 3, 552.

Lee, C.P. and Ernster, L. (1968) Eur. J. Biochem. 3, 391-400.

Lein, S. and Racker, E. (1971) Methods in Enzymology 23, 547.

Libbertini, L.J., Waggoner, A.S., Jost, P.C. and Griffiths, O.H. (1969) Proc. Nat. Acad. Sci. U.S. 64, 13.

Linnane, A.W., Saunders, G.W., Gingold, E.B. and Lukins, H.B. (1968) Proc. Nat. Acad. Sci. U.S. 59, 903.

Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265.

MacLennan, D.H. and Tzagoloff, A. (1968) Biochem. A.C.S. 7, 1603.

Masamure, S., Sehgal, J.M., Van Temela, E., Strong, F. and Peterson, W.H. (1958) J. Am. Chem. Soc. 80, 6092.

Melhorn, K. in "Membrane Molecular Biology" Eds. Fox, C. and Keith, A. p. 972. Sinauer Associates Inc. Stamford, Conn.

Mitchell, P. in "Chemiosmotic Coupling in Oxidative Phosphorylation and Photosynthetic Phosphorylation" Glynn Res. Lab., Bodmin, Cornwall, U.K.

Mitchell, P. (1973) FEBS Lett. 33, 267.

Mitchell, P. (1974) FEBS Lett. 43, 189.

Munoz, E., Salton, M.J.R., Ng, M.H. and Schor, M.T. (1969) Eur. J. Biochem. 7, 490.

Murray, D. and Linnane, A.W. (1972) Biochem. Biophys. Res. Commun. 49, 855-862.

Oldfield, E., Chapman, D. and Derbyshire, W. (1971) FEBS Lett. 16, 102.

Parsons, D.F. and Yano, Y. (1967) Biochim. Biophys. Acta 135, 362-364.

Penefsky, H. (1965) J. Biol. Chem. 240, 4695-4702.

Perlman, P.S. and Mahler, H.R. (1970a) Arch. Biochem. Biophys. 136, 245

Perlman, P.S. and Mahler, H.R. (1970b) Bioenergetics 1, 113

Perlman, P.S. and Mahler, H.R. (1971) Nature New Biology 231, 12

Pullman, M.E., Penefsky, H.S., Datta, A. and Racker, E. (1960) J. Biol. Chem. 235, 3322-3329.

Racker, E. (1969) in "Membrane Proteins" Procedings of a

Symposium sponsered by the New York Heart Assoc. Little, Brown and Co. Boston. p. 38

Rhodes, A., Fantes, K.H., Boothroyd, B., McGonegle, M.P. and Crosse, R. (1961) Nature (Lond.) 192, 952.

Roberton, A.M., Holloway, C.T., Knight, I.G. and Beechey, R.B. (1968) Biochem. J. 108, 445-456.

Rohm, . and Haas, . "Surfactants - Handbook of Physical Properties" p. 7. Rohm and Haas, Philadelphia, Pa.

Roodyn, D.B. and Wilkie, D. (1968) in "The Biogenesis of Mitochondria" Methuen and Co., Ltd., London.

Rose, M.S. and Aldridge, W.N. (1972) Biochem. J. 127, 51.

Schnebli, H.P., Abrams, A.P. (1970) J. Biol. Chem. 245, 1115.

Selwyn, M.J., Dawson, A.P., Stockdale, M. and Gain, N. (1970) Eur. J. Biochem. 14, 120.

Senior, A.E. and Brooks, J.C. (1970) Arch. Biochem. Biophys. 140, 257.

Shannon, C., Enns, R., Wheelis, L., Burchiel, K. and Criddle, R.S. (1973) J. Biol. Chem. 248, 3004-3011.

Schatz, G. (1967) J. Biol. Chem. 242, 2552-2560.

Shaw, P. (1967) Antibiot. 1. Springer, Verlag. Eds. Gottleib, D. Shaw, P.D. p. 585.

Shimshick, J. and McConnell, H. (1973) Biochem. Biophys. Res. Commun. 53, 446-451.

Slater, E.C. (1969a) Terwells. "Inhibitors as Tools in Cell Research" Springer Verlag.

Slater, E.C. (1969b) Ibid. p. 528.

Smith, R.M., Petersen, W.H. and McCoy, E. (1954) Antibiotics and Chemotherapy IV 962.

Somlo, M. and Kruppa, M. (1974) Biochem. Biophys. Res. Commun. 59, 1165.

Sone, N. and Hagihara; B. (1964) J. Biochem. (Tokyo) 56, 151.

Stekhoven, F.S., Waitkus, R.F., and Van Moerker ,H.T. B (1972) Biochem. 11, 1144.

Stuart, R.D. (1970) Biochem. Biophys. Res. Commun. 89, 1045.

Sund, H. (1971) in "New Techniques in Amino Acid Peptide and Protein Analysis" Chapter 10, p. 413-416. Ann Arbor Science Publishers Inc.

Swanljung, P., Swanljung, H. and Partis, M.D. (1972) Biochem. J. 128, 479-480.

Swanljung, P., Frigeri, K., Ohlson, K. and Ernster, L. (1973a) Biochim. Biophys. Acta 305, 519

Swanljung, P. (1973b) Anal Biochem. 43, 382-387.

Thompson, R.Q., Hoen, M.M. and Higgins, C.E. (1961) in "Antimicrobial Agents and Chemotherapy" p. 476.

Tzagoloff, A., Byington, K. and MacLennan, D. (1968a) J. Biol. Chem. 243, 2405

Tzagoloff, A., MacLennan, D. and Byington, K. (1968b) Biochem. 7, 1596.

Tzagoloff, A. (1969a) J. Biol. Chem. 244, 5020-5026.

Tzagoloff, A. (1969b) J. Biol. Chem. 244, 5027

Tzagoloff, A. (1970) J. Biol. Chem. 245, 1545.

Tzagoloff, A. and Meagher, P. (1971 ) J. Biol. Chem. 246, 7328-7326.

Tzagoloff, A. (1971b) J. Biol. Chem. 246, 3050-3056.

Tzagoloff, A. and Meagher, P. (1972 ) J. Biol. Chem. 247, 594-603.

Tzagoloff, A. (1972b) J. Biol. Chem. 247, 6511-6516.

Tzagoloff, A. and Akai, A. (1972c) J. Biol. Chem. 247, 6517-6523.

Tzagoloff, A., Rubin, M. and Sierra, M. (1973) Biochim. Biophys.

Tzagoloff,A.,Akai,A.,Needleman,R.(1975) Proc.Nat.Acad.Sci.USA 72, 2054 - 2057.

Acta Rev. on Bioenergetics. 301, 71-104.

Vambutas, V. and Racker, E. (1970) in "Membranes of Mitochondria and Chloroplasts" Ed. Racker, E. p. 127 Van Nostrand Rheinhold, Co., N.Y.

Wakabayashi, R. and Gunge, N. (1971) FEBS Lett. 6, 302

Wakabayashi, R. (1971) Antibiot. Tokyo 25, 475.

Walter, P., Lardy, H.A. and Johnson, D. (1967) J. Biol. Chem. 242, 5014-5018.

Weber, R. and Osborn, M. (1969) J. Biol. Chem. 244, 4406.

Wilkie, D., Saunders, G. and Linnane, A.W. (1967) Genet. Res. Camb. 10, 199.

Wilkie, D. (1970) J. Mol. Biol. 47, 107

Wintersberger, E. (1965) Biochem. Z. 341, 409.

# APPENDIX

## A PREPARATIVE GEL ELECTROPHORESIS APPARATUS

This short appendix describes an attempt to design and produce a Preparative Gel Electrophoresis Apparatus for use in further purification of the mitoribosomal subunits of the O.S.ATPase. The work was undertaken mainly in view of the considerable expense of such commercially available apparatus. A further consideration was that the apparatus needed to be capable of analysing approximately 2 mgrms of protein to provide sufficient mitoribosomal subunits for further structural studies. Extrapolating from work with analytical gels a preparative gel of the dimensions indicated in Figure A2 should be capable of analysing upto 2.3 mgrms of O.S.ATPase .At the time of starting this investigation no commercial product could be found which would analyse this quantity of protein. The assembled apparatus is shown in Figure A1; it consists basically of separate copper cathode buffer reservoir and lower anode buffer reservoir. The upper cathode electrode is the electrode from a standard Shandon Analytical Electrophoresis Apparatus ( Shandon Southern, Camberley, Surrey ), the lower anode is a short length of platinum wire soldered to copper wire, the copper and solder subsequently being encased in teflon tubing to prevent electrolysis of the copper. The polyacrylamide gel was cast in the detachable gel tube which was a push fit into the bottom of the upper buffer reservoir. The gel tube was furnished with a collar to prevent any possible slippage. The gel tube itself was a push fit into the elution chamber and was scaled by an 'O' ring inset into the side of the elution chamber. The elution chamber, shown twice actual size in Figures A2 and A3, consisted of upper and lower flanges held together by three plastic coated bolts. Between the two flanges was sandwiched a rubber washer 1 mm in thickness, the purpose of which was to hold in place and prevent leaks from a piece of dialysis tubing, shown at C in Figure A1, which formed the bottom of the elution chamber.

Elution of the electrophoresed protein from the chamber was via a large bore syringe needle at B., replacement buffer being drawn in at the three smaller bore needles at A. Tests with dyes and cytochrome C indicated that a complete change of buffer in the elution chamber could be achieved with a volume of eluate 2 to 3 times the volume of the elution chamber.

Initial testing of the apparatus was carried out using cytochrome C, the electrophoresis of which could be monitored visually. After prolonged electrophoresis it was found that the upper buffer reservoir became depleted of SDS, the conditions of electrophoresis being essentially those described by Weber and Osborne, (Weber K., Osborne M. 1969) . It was therefore found necessary to have continuous mixing of cathode and anode buffers. The direction of buffer flow was as indicated by the arrows in Figure A1, the flow rate was controlled so that the buffer dripped at outlets X, Figure A1, thus preventing any short circuiting around the gel. The buffer in the lower reservoir was slowly stirred with a magnetic stirrer. This was found to exercise a substantial cooling effect on the gel and made possible the use of higher currents. SDS treated catalase, aldolase, trypsin and cytochrome C were electrophoresed as a mixture, the gel was sliced longitudinally and stained. The peptide bands were well separated and there was no evidence of streaking due to wall effects or the use of excessive currents, the gel being subjected to a current of 160 milliamps.

Bromophenol blue and cytochrome C were successfully electrophoresed and eluted , using electrophoresis buffer as the eluate. With non-visual monitoring of the eluate one is faced with the problem of having to take small fractions at fairly rapid flow rate to avoid cross contamination of the bands over a long time course. This procedure was found to be logistically unpracticable and also led to substantial dilution of small amounts of protein. It was decided, therefore, to attempt to attach a visual label to the peptides of the OS ATPase, so that it was necessary to elute from the chamber only when peptides were seen to enter it. The production of peptides labelled using such compounds as dansyl chloride and fluorescamine, which enable

them to be located visually, usually results in a peptide with an unreactive amino end group and so are of restricted use in structural studies. In view of this it was decided that only 10% of the protein applied to the gel should be labelled in this way. Upon electrophoresis the ragged O.S.ATPase peptides of high molecular weight, i.e. those of cytoribosomal origin, could be clearly distinguished, as could subunit 9, the major mitoribosomal product, but the other mitoribosomal products were present in such small quantities and the labelled peptides in even smaller quantities, that it proved impossible to locate them with any degree of certainty. The apparatus described here, however, gives sufficient resolution of unmeasured bands in sufficient quantities; the remaining problem is one of location of the bands. Perhaps an enrichment procedure, such as those suggested in the General Discussion, for the mitoribosomal products would greatly assist in a visual location of these bands.

FIGURE A.1

Preparative Gel Electrophoresis Apparatus.

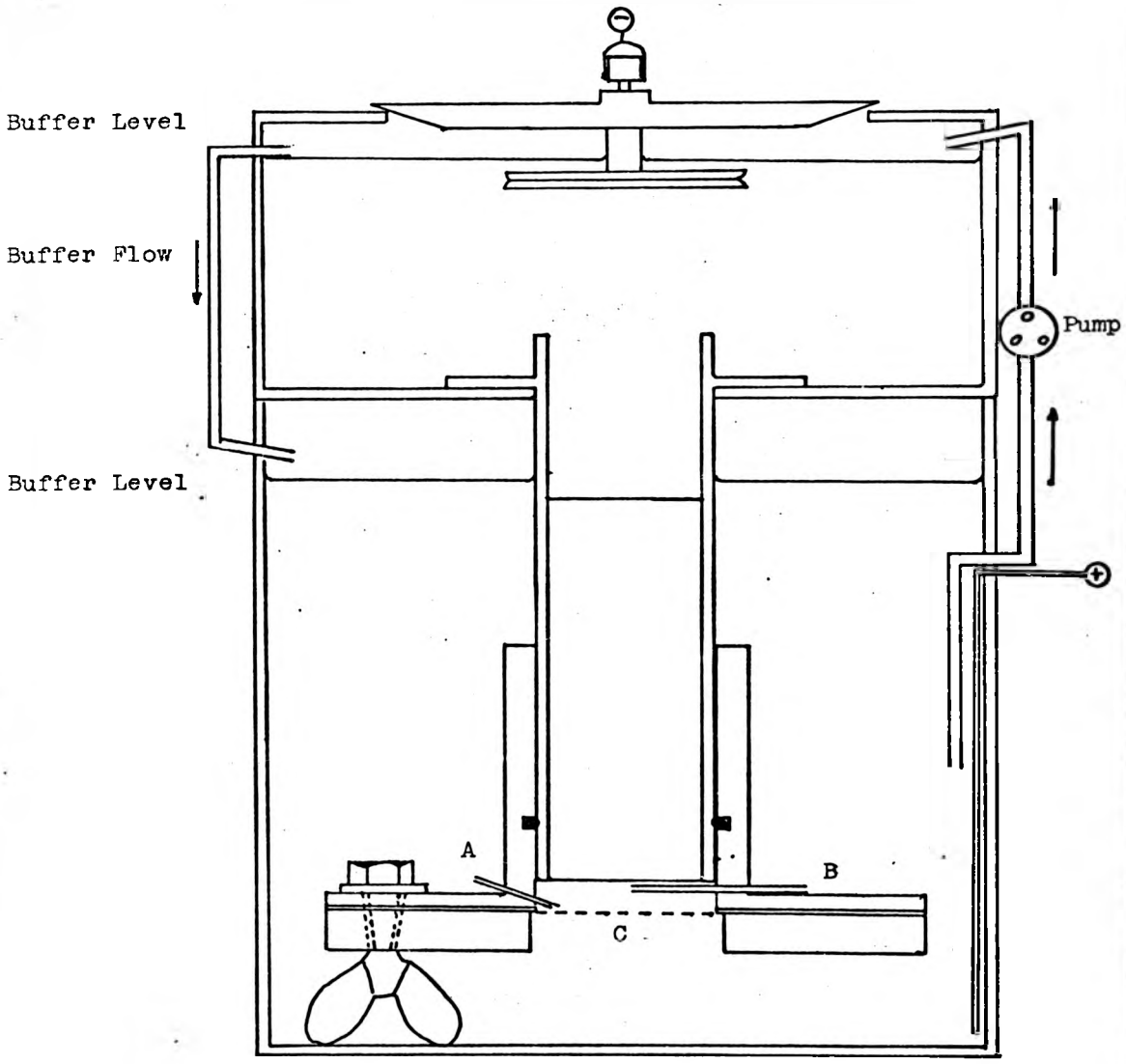
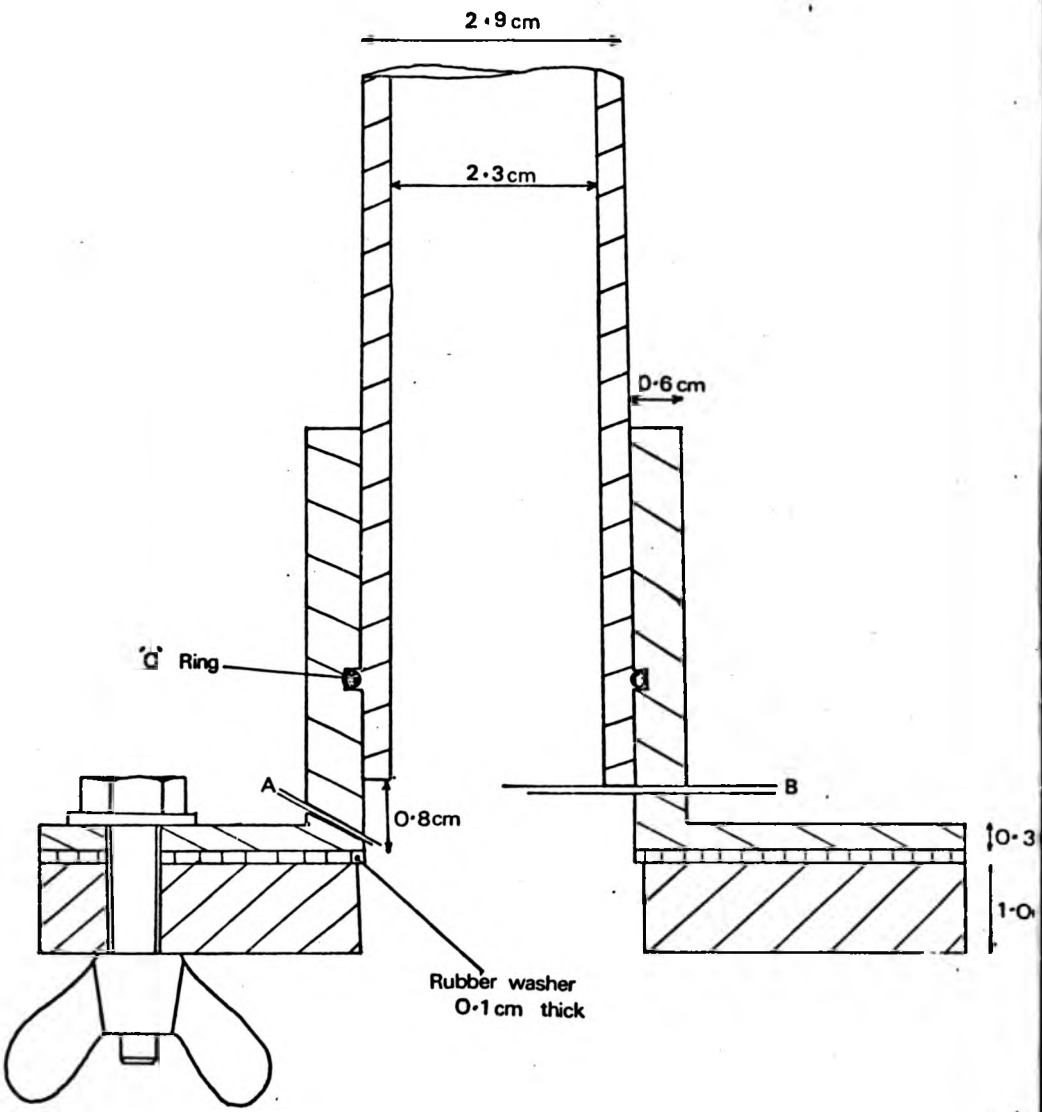


FIGURE A.2

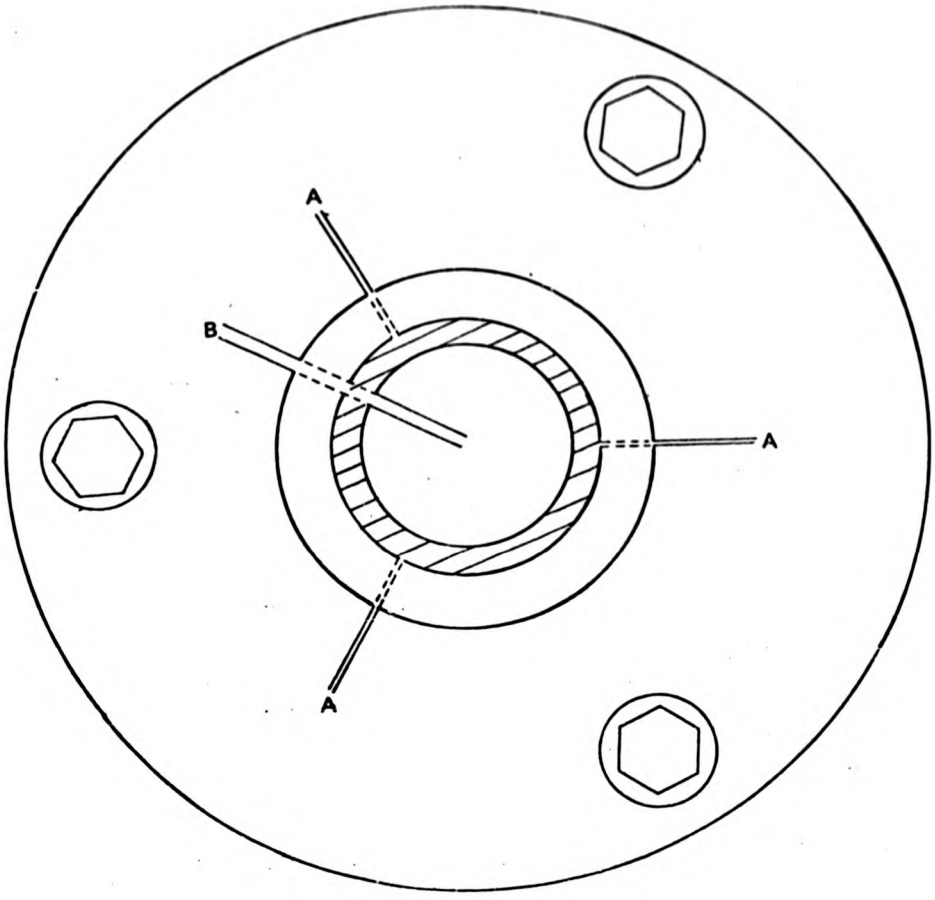
Preparative Gel Elution System




ELEVATION

A3

FIGURE A.3




PLAN