Oligomycin Resistant Mutants of *Saccharomyces Cerevisiae* : The Class Structure

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CONTENTS

ABBREVIATIONS

ad^- / ad_2^+	:	Auxotrophic / prototrophic for 'adenine two' determinant
ADP	:	Adenosine diphosphate
Ant A	:	Antimycin A
arg^- / arg^+	:	Auxotrophic / prototrophic for 'arginine' determinant
ATP	:	Adenosine triphosphate
Aur	:	Aurovertin
CAP	:	Chloramphenicol
Chloro CCP	:	Chloro carbonyl cyanide phenylhydrazone
Cit P	:	Citrate phosphate buffer
$CoSO_4$	:	Cobalt sulphate
C^R / C^S	:	Chloramphenicol resistant / sensitive determinant
DCCD	:	Dicyclohexylcarbodiimide
DNP	:	Dinitrophenol
E^R / E^S	:	Erythromycin resistant / sensitive determinant
FCCP	:	Fluoromethoxy carbonyl cyanide phenylhydrazone
Glut	:	Glutaraldehyde
HNO_2	:	Nitrous acid
$KMnO_4$	:	Potassium permanganate
met^- / met^+	:	Auxotrophic / prototrophic for methionine determinant
Mika	:	Mikamycin
Octyl DNP	:	Octyl dinitrophenol

OL^R/OL^S	:	Oligomycin resistant/sensitive determinant (Nomenclature of this laboratory)
$OL^R I/OL^R II$	:	Oligomycin resistant determinants of class I/class II mutants
O^R/O^S	:	Oligomycin resistant/sensitive determinant (Nomenclature of Slonimski's group)
OsO_4	:	Osmium tetroxide
$P_i (H_3PO_4)$	:	Inorganic phosphate
P	:	Phosphate buffer
RNAase	:	Ribonuclease
S^R/S^S	:	Spiramycin resistant/sensitive determinant
TET	:	Triethyltin
TTFB	:	Tetrachlorotrifluoromethyl benzimidazole
U.V.	:	Ultraviolet light
Y_s	:	Molar Growth yield
ρ^-	:	rho minus or cytoplasmic 'petite' determinant
ρ^+	:	rho plus or 'grande' determinant
$\omega^+, \omega, \omega^n$	:	Allelic forms of the mitochondrial sex factor

Addenda to Abbreviations List.

CP^R/CP^S	:	Chloro carbonyl cyanide phenylhydrazone resistant/sensitive determinant.
Cy^R/Cy^S	:	Cycloheximide resistant/sensitive determinant.
E^R(Slonimski)	=	Er^R(Avner's nomenclature).
S^R(Slonimski)	=	Sp^R(Avner's nomenclature).

SUMMARY

A series of oligomycin resistant mutants has been isolated following u.v. irradiation. The phenotypic and genotypic properties of these mutants, which show high levels of resistance to oligomycin and rutamycin, have been investigated and, on the basis of these results, it has been possible to divide the mutants into two main classes, class I and class II.

Class I

Mutants of this class show low levels of cross resistance (increases of two- to four-fold) to various uncouplers such as TTFB, 1799 and chloro CCP, to inhibitors of oxidative phosphorylation like aurovertin and triethyltin, to the inhibitor of electron transport, antimycin A, and protein synthesis inhibitors such as cycloheximide, mikamycin, chloramphenicol and erythromycin. The mutants are apparently, however, resistant to neither DNP nor octyl DNP.

In a high percentage of the mutants, exposure to low temperatures ($20^{\circ}C$) resulted in the loss of the primary resistance to oligomycin and rutamycin, though the secondary cross resistances were apparently unaffected. No effect of either high or low temperatures on either the fermentable or non-fermentable growth of these mutants was apparent.

No alteration in the growth rate of the majority of the strains or in their appearance on electron microscopic examination was detected, though their growth yield is 15 - 20% lower than the wild type strain. Genetic analysis has revealed that this class of mutant clearly differs genotypically as well as phenotypically from the class II mutants and moreover, had indicated that the determinant controlling oligomycin resistance is a nuclear gene. No evidence is apparent to support the concept that these are multi-gene mutants. The genetics of these mutants exhibited many anomalous features, which remain to be explained, many of the experiments in this thesis describing, rather than explaining the phenomena observed.

Class II

The mutants in this class show resistance only to oligomycin and rutamycin. No cold or heat sensitivity of growth was observed and only one mutant in this

class showed any temperature sensitivity with regard to its oligomycin resistance. No alteration in the growth rate, growth yield or mitochondrial morphology of any of the mutants in this class was found.

Genetic analysis of these mutants revealed that all the mutants tested showed cytoplasmic inheritance, the genetic determinant being located on the mitochondrial DNA. Allelism tests have demonstrated that genotypically the class is not homogeneous and the mutants were sub-divided into two non-allelic recombination groups. The genetic determinants concerned in each sub-group were confirmed to lie on mDNA.

CHAPTER 1

INTRODUCTION

General Introduction

Despite an intensive experimental effort for many years now, the nature of the membrane complexes concerned in mitochondrial energy conservation remains, in many particulars, as obscure as ever. Essentially three theories of oxidative phosphorylation and variants on them provide the keystone for experiments concerning this metabolic process. These are the chemiosmotic (or Mitchell) hypothesis, the chemical hypothesis and the conformational hypothesis. The former postulates that the initial event of oxidative phosphorylation is essentially a charge separation across a membrane driven by electron transport, the second takes as its model glycolytic substrate level ATP generation and postulates that the primary event involves energy conservation in a yet undiscovered and uncharacterised high energy chemical compound, whilst the last postulates that either macro or micro changes in the spatial orientation of the mitochondrial inner membrane results in this energy conservation. The evidence both for and against each of these theories has been recently summarised by Slater (Slater, 1971) but is, however, far from definitive. Whilst some biochemical information is available about the enzymes concerned in the terminal steps of oxidative phosphorylation, the F_1 and ATPase activities of the mitochondrion, essentially nothing is known about the earlier steps concerning this process, and there is a complete lack of information concerning the components involved in the primary conservation step or steps.

In view of this situation and the rapid development of organelle genetics, a concerted genetic and biochemical approach to the problems of the structure and function of the energy conservation complex(es) seemed attractive, provided that specific mutants relating to this process could be isolated. Isolation of such mutants may provide information about the biogenesis of the mitochondrion and about the complex interactions occurring between the mitochondrion, cytoplasm and nucleus.

The isolation of mutants with specific lesions in their oxidative phosphorylation processes can be pursued in at least two ways. Firstly, by isolation of mutants

unable to grow on non-fermentable substrates such as glycerol, which, however, are able to respire and have retained functional electron transport. The phenotype of such mutants resembles an uncoupled mitochondrion. Secondly, the isolation of mutants resistant to specific inhibitors and uncouplers of oxidative phosphorylation such as oligomycin, DNP, chloro CCP, etc.

This thesis describes such a genetic approach to the problems of oxidative phosphorylation and recounts attempts to isolate specific mutants of S.cerevisiae using both the approaches detailed above. In particular, this thesis records the results of the phenotypic and genotypic characterisation of mutants isolated as resistant to the inhibitor oligomycin.

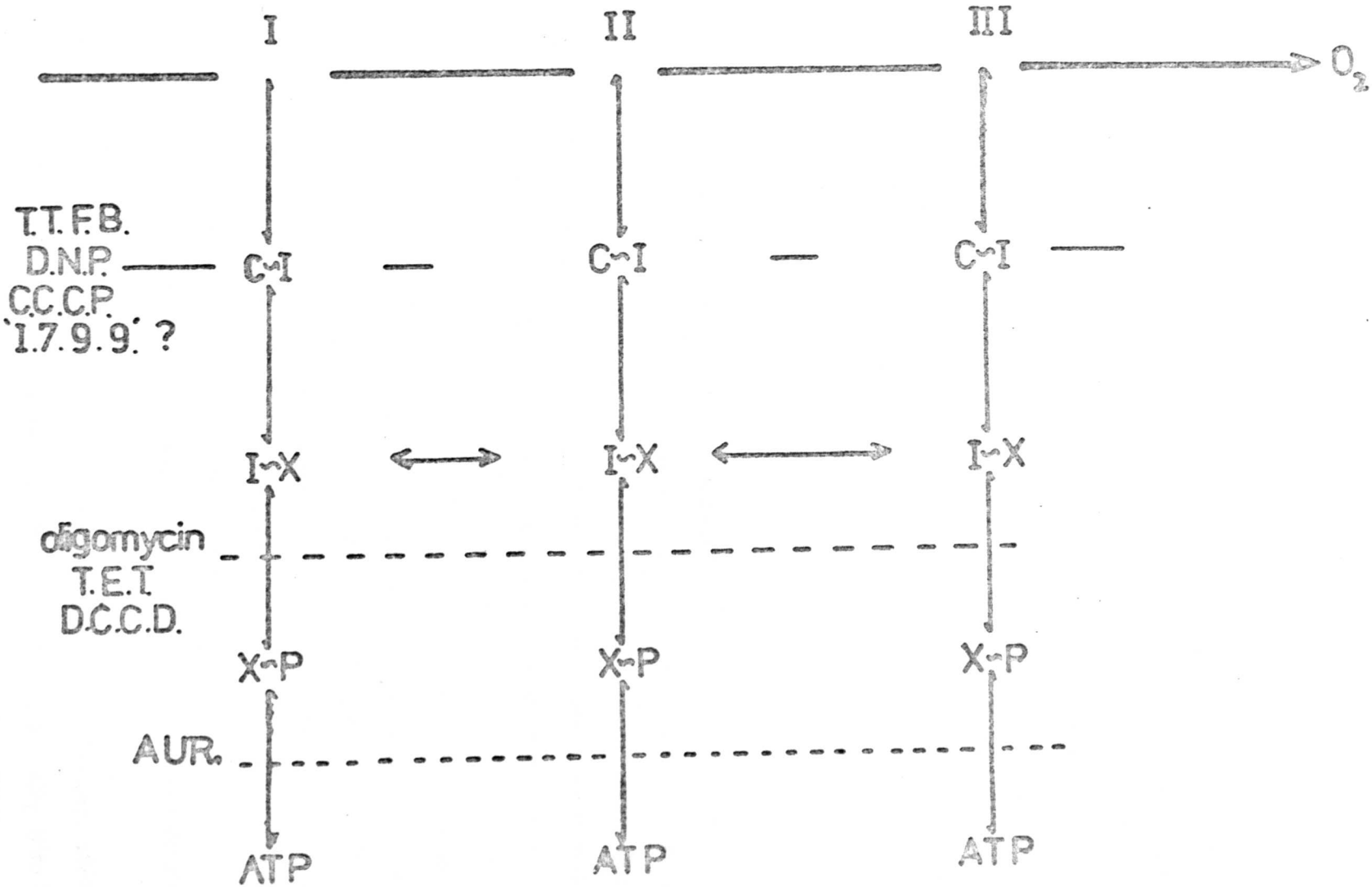
Inhibitors and Uncouplers of Oxidative Phosphorylation Used in these Studies

Oligomycin and Rutamycin

Exhaustive reviews of the properties of these compounds have recently been presented by Shaw (Shaw, 1967) and Slater and Ter Welles (Slater and Ter Welles, 1969). Both oligomycin and rutamycin are antibiotics 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). Oligomycin itself is composed of three structurally related alcohols, oligomycins A, B and C which vary in their molecular weight from 400 to 480 (Masamune et al., 1958; Thompson et al., 1961). Of the oligomycins and rutamycin, oligomycin A appears the most effective inhibitor, the order of effectiveness of the oligomycins as inhibitors of coupled respiration A > B > C (Lardy et al., 1964) being the same order as reported by Marty et al. (Marty and McCoy, 1959) for the effectiveness of the oligomycins in the inhibition of the growth of various fungi. The complete chemical structure of oligomycin has recently been reported (Von Glehn et al., 1972). Oligomycin acts as an inhibitor in eucaryotic cells by interfering with mitochondrial metabolism (Shaw, 1967) and this inhibitory action shows a high degree of specificity. There is some evidence showing that oligomycin can act on the cell membrane ($Na^+ + K^+$) stimulated ATPase and interfere with cellular ion transport (Van Rossum, 1964; Whittam et al., 1964) but higher concentrations of oligomycin are needed to interfere with these reactions than are necessary to affect

FIGURE 1.1.

Schematised diagram of the site of action of inhibitors and uncouplers of oxidative phosphorylation


I
II
III
O_2
T.T.F.B.
D.N.P.
C.C.C.P.
'1.7.9.9.'?
C~I
C~I
C~I
I~X
I~X
I~X
oligomycin
T.E.T.
D.C.C.D.
X~P
X~P
X~P
AUR.
ATP
ATP
ATP

mitochondrial metabolism. Whilst there is agreement that oligomycin acts primarily on the mitochondrion in vivo, its degree of specificity for the Mg^{2+} mitochondrial ATPase rather than the Na^{+} - K^{+} ATPase is a matter of some dispute (Contessa and Bruni, 1971 ; Slater and Ter Welles, 1969 ; Tobin and Slater, 1965 ; Whittam et al., 1964 ; Van Croningen and Slater, 1963).

The effect of oligomycin on the mitochondrion is to inhibit coupled respiration (Huijing and Slater, 1961), to inhibit mitochondrial ATPase activity regardless of the activating agent, providing that the F_1 remains membrane-bound, to inhibit the ^{32}Pi - ATP, ADP - ATP , $H_3P^{18}O_4$ - H_2O exchange reactions and to prevent all energy-linked reversal reactions from occurring when ATP is the energy source. No inhibition of the reversal reactions is apparent when energy is conserved by respiratory chain substrate oxidation.(Danielson and Ernster, 1963 ; Chance, 1961 ; Lardy et al., 1964 ; Wadkins and Lehninger, 1963a ; Wadkins and Lehninger, 1963b). Oligomycin similarly does not affect mitochondrial ion transport when energy is supplied by substrate oxidation (Brierly et al., 1962).

The effects of oligomycin on sub-mitochondrial particles are slightly more complex than that shown on mitochondria and depend not only on the concentration of oligomycin used, but also on the type of particle. Certain types of particle, although showing an inhibition of phosphorylation by oligomycin at high concentrations identical to that exhibited by the intact mitochondrion, show, when lower concentrations of oligomycin are used, a stimulation of phosphorylation (Lee and Ernster, 1965 ; Lee and Ernster, 1968). Low concentrations also result in a stimulation of the energy-dependent transhydrogenase activity catalysed by these particles. This oligomycin stimulating effect is seen only with particles partially deficient in certain phosphorylating factors and no effect is observed when particles that are totally deficient of F_1 are used (Racker and Horstmann, 1967). It has been suggested that this effect is due to oligomycin promoting a membrane conformation favouring the mutual accessibility of the different components of the phosphorylating system (Slater and Ter Welles, 1969) whilst excluding the entry of agents that can dissipate conserved energy.

The isolated F_1 or ATPase itself is oligomycin insensitive (Racker, 1965). When F_1 is bound to a specific F_1 depleted membrane fraction TUA - CF_o with F_c, the ATPase activity itself is inhibited (Bulos and Racker, 1968) but addition of

phospholipid restores the activity and this activity is then oligomycin sensitive. The second factor called F_c (Bulos and Racker, 1968) or O.S.C.P. (MacLennan and Tzagaloff, 1968) is necessary for F_1 binding to TUA - CF_0 particles.

Oligomycin has been shown to penetrate tissue slices and inhibit respiration in such tissue (Wu, 1964; Tobin and Slater, 1965) and has also been reported to inhibit yeast cell respiration (Kovác et al., 1970). However, some doubt remains as to the effectiveness of oligomycin inhibition of respiration in intact yeast cells in short-term experiments (Somlo, private communication).

Oligomycin has no effect on photophosphorylation at concentrations which affect oxidative phosphorylation but at higher concentrations (> 10.0 γ/ml) it acts as a weak uncoupling agent (Avron and Shavit, 1965).

DCCD (Dicyclohexylcarbodiimide)

DCCD has been shown by Beechey and coworkers (Beechey et al., 1967; Roberton et al., 1968; Beyer et al., 1969) to act in an essentially similar manner to oligomycin in inhibiting mitochondrial oxidative phosphorylation and the associated mitochondrial reactions such as the $H_3P^{18}O_4$ - H_2O and ADP - ATP exchange activities, ATPase activity and ATP driven energy-linked reversal reactions. Like oligomycin it has been shown to stimulate oxidative phosphorylation in sub-mitochondrial particles (Racker and Horstmann, 1967). DCCD, unlike oligomycin however, apparently permeates mitochondria and sub-mitochondrial particles only slowly and pre-incubation is necessary if inhibition is to be observed with low concentrations of DCCD (Beechey et al, 1967). Using such a procedure, inhibition of mitochondrial phosphorylation can be observed with concentrations of DCCD of about 1 n mole/mg protein. DCCD, once bound to sub-mitochondrial particles, is not, unlike oligomycin, removed by washing with phospholipids (Beechey et al., 1967) and it has been postulated that DCCD acts by forming covalent bonds to reactive groups at the actual site of inhibition. Beechey and coworkers (Beechey et al., 1967; Cattell et al., 1970) have succeeded in extracting a proteolipid fraction(s) containing bound C^{14}DCCD from sub-mitochondrial particles incubated with this substance, and the majority of the bound DCCD was found to elute on chromatography as a single peak.

The supposition that DCCD is bound covalently when inhibiting oxidative phosphorylation is challenged by the findings of Bruni et al. (Bruni et al., 1971) who have shown that both oligomycin and DCCD inhibition can be relieved by the addition

of phospholipids. These experiments were, however, essentially short-term experiments and the effects of the phospholipids are probably due solely to straightforward partition effects on these lipophilic inhibitors. The results of these experiments suggest that DCCD can inhibit without binding covalently to the mitochondrion, even if normally it is covalently bound.

DCCD differs from oligomycin in its action in a number of ways. DCCD is, for instance, capable of inhibiting bacterial phosphorylation and bacterial ATPase activity though, as with oligomycin inhibition of mitochondrial ATPase activity, this inhibition occurs only when the F_1 is still integrated with the membrane. Oligomycin on the other hand is without effect on bacterial systems. A second difference between the two compounds has been shown by Kovác et al. (Kovác et al., 1968b) who found that DCCD inhibited the respiration of intact yeast cells under conditions where oligomycin was relatively ineffective. The difference is, in this instance, presumably related to differences in cell permeability towards the two substances. Reviews of the carbodiimides have shown that water soluble derivatives are relatively ineffective in inhibiting mitochondrial activities (Abrams and Baron, 1970), only hydrophobic carbodiimides being active inhibitors.

Aurovertin

Aurovertin is an antibiotic which has been isolated from Calcarisporium arbuscula (Preiss). It has been shown to be an effective inhibitor of oxidative phosphorylation, giving an identical titration curve to oligomycin for inhibition of oxidative phosphorylation, ^{32}Pi - ATP exchange and the exchange of ^{18}O between Pi and H_2O in rat liver mitochondria (Lardy et al., 1964). Although the inhibitory effects of aurovertin and oligomycin on the exchange reactions and oxidative phosphorylation are additive in intact mitochondria (Lardy et al., 1964; Lee and Ernster, 1968), aurovertin, unlike oligomycin, does not augment the phosphorylation of sub-mitochondrial particles prepared by sonication in the presence of EDTA (Lee and Ernster, 1968). Indeed, aurovertin has even been observed to abolish the stimulation caused by oligomycin (Lee and Ernster, 1968). Additional evidence as to the differences in action of oligomycin and aurovertin is provided by the finding that aurovertin, unlike oligomycin, does not inhibit the mitochondrial ATPase activity induced by a number of activators such as selenate and deoxycholate (Lardy et al., 1964) and was much less effective than oligomycin in inhibiting the

DNP stimulated ATPase. In addition, aurovertin failed to inhibit ATP driven mitochondrial swelling and contraction (Connelly and Lardy, 1964), had little or no effect on ATP driven transhydrogenation reactions and only inhibited the ATP mediated reduction of NAD by succinate by 43% when concentrations of aurovertin sufficient to abolish phosphorylation at all three sites were used (Lenaz, 1965). ATP supported Ca^{2+} transport into mitochondria is also blocked by oligomycin but not by aurovertin (Bielawski and Lehninger, 1966).

To summarise, therefore, aurovertin, unlike oligomycin, appears to be an effective inhibitor of only the forward reactions of oxidative phosphorylation and a much less effective inhibitor of the ATP driven reversal reactions. Mitchell and Moyle have proposed that in mitochondria, aurovertin reacts with a protein which controls the adenine nucleotide affinities whilst oligomycin reacts with another enzyme which controls the maximal rate of the phosphorylating system. They envisage that aurovertin interacts with a protein to affect the affinities of the ATP synthesising enzymes for ATP and ADP, the affinity for ADP being decreased whilst that for ATP is increased. Thus, ATP synthesis which requires ADP binding is inhibited by aurovertin whilst ATP hydrolysis is increased, because the presence of aurovertin results in less hindrance by the competitive inhibitor ADP (Mitchell and Moyle, 1970).

Aurovertin is thought to inhibit phosphorylation at a site farther from the respiratory chain than is the inhibition site of oligomycin (Lee and Ernster, 1968), acting on a step between $X \sim P$ and ATP formation, whilst oligomycin inhibits between the $1 \sim X$ and $X \sim P$ steps (Figure 1.1). Lee and Ernster discount the scheme proposed by Lardy et al. that the two inhibitors act on alternative pathways (Lardy et al., 1964). Aurovertin has been shown to bind to both purified F_1 and liver mitochondrial ATPase to form a highly fluorescing complex (Lardy et al., 1964; Lardy and Lin, 1966). Fractions devoid of F_1 do not form this complex.

Organotins, Including Triethyltin

Triethyltin and other trialkyltins affect the mitochondrion in two distinct ways. Firstly, they are potent inhibitors of coupled respiration and of phosphate esterification of respiring mitochondria (Aldridge, 1958; Aldridge and Cremer, 1955; Aldridge and Threlfall, 1961; Sone and Hagihara, 1964). In this respect they appear to behave somewhat like oligomycin, being capable of inhibiting not only oxidative phosphorylation, but also partial reactions such as the $^{32}P_i$ - ATP

exchange reaction and mitochondrial ATPase activity (Aldridge and Street, 1964; Aldridge, 1958; Aldridge and Threlfall, 1961). Tzagaloff et al. (Tzagaloff et al., 1968) and Byington (Byington, 1971) using respectively tri-n-butyltin chloride and triphenyltin chloride, have observed oligomycin-like inhibition of the ATPase activity of beef heart sub-mitochondrial particles. Byington has concluded that triphenyltin acts in a highly specific manner to form a stable complex with the rutamycin (oligomycin D) binding site in sub-mitochondrial particles.

Aldridge and Street (Aldridge and Street, 1970) using ^{113}Sn labelled trimethyl and triethyltin have, in fact, succeeded in preparing a fraction containing only 15% of the original mitochondrial protein which still contains bound, the labelled inhibitor. There is, however, some doubt as to whether the site of action of the trialkyltins and oligomycin are identical, as Aldridge found that triethyltin inhibited DNP stimulated mitochondrial respiration (Aldridge, 1958; Aldridge and Street, 1971). This led Aldridge to postulate that DNP and triethyltin have the same site of action. Sone and Hagihara (Sone and Hagihara, 1964) using tri-n-butyltin chloride were, however, unable to confirm this result and failed to find any inhibition of DNP stimulated respiration when the concentrations of the trialkyltin employed were sufficient to inhibit coupled respiration. They did, however, find some inhibition after a time lag when high concentrations of the compound were used.

Triethyltin also differs from oligomycin both in the speed of the onset of inhibition and in the more potent inhibition of coupled respiration it shows when succinate or pyruvate are substrates rather than ascorbate. Oligomycin acts equally effectively against coupled respiration regardless of the substrate (Aldridge and Street, 1971).

Secondly, and in addition to its inhibitory action on mitochondrial phosphorylation, trialkyltins such as triethyltin have been found to uncouple mitochondrial respiration and induce mitochondrial ATPase activity (Aldridge, 1958). Trialkyltin compounds have also been shown to cause marked mitochondrial swelling under certain conditions (Aldridge and Street, 1964; Stockdale et al., 1970). Stockdale et al. (Stockdale et al., 1970) have succeeded in separating the two effects of the trialkyltin compounds. They found that when mitochondria were incubated in sucrose medium in the absence of permeating anions, only the oligomycin-like effects of the trialkyltin compounds were observed; that is, inhibition of coupled

respiration and of arsenate stimulated respiration. No inhibition of other uncoupler stimulated respiration was observed and neither was mitochondrial swelling. Incubation of mitochondria with trialkyltins in the presence of permeant anions such as contained in KCl buffers resulted, on the other hand, not only in the aforementioned effects but, in addition, some release of respiratory control and marked mitochondrial swelling. A time dependent inhibition of uncoupler stimulated respiration was also apparent and they claim that this is a secondary effect of the extensive swelling. Uncoupling by trialkyltin compounds is probably due to the action of the anion-hydroxyl exchange reaction which trialkyltin compounds appear to be able to mediate (Selwyn et al., 1970a; Selwyn et al., 1970b) followed by leakage of the anion from the mitochondrion, this resulting in discharge of both the pH and membrane potentials. Manger has observed that triethyltin chloride inhibits intra-mitochondrial accumulation of substrate anions (Manger, 1969) and this effect is presumably related to the discharge of the pH gradient and accumulation of chloride ion within the mitochondrion caused by the trialkyltin mediated anion-hydroxide exchange. As substrate anions generally enter indirectly in exchange for hydroxyl ions (Chappell, 1968) this would be expected to lead to the reduction of substrate anion accumulation observed.

Aldridge and Street (Aldridge and Street, 1971) have examined the effects of triethyltin on mitochondrial processes and have shown that some of the mitochondrial functions are very much more sensitive to triethyltin than others. Both the uncoupling activity of triethyltin and its inhibition of ascorbate linked respiration are much less sensitive to triethyltin than, for example, pyruvate linked respiration. Three alternative explanations of this data have been considered. Firstly, that there is only one triethyltin inhibition binding site, but that it is a complex one, progressive saturation of a number of sites leading to the different inhibition and uncoupling characteristics. Secondly, that there are two binding sites with different binding affinities. Thirdly, that there is only one binding site, some of the inhibitory effects being due to triethyltin which is not bound. Aldridge and Street have concluded that their binding data is incompatible with the second hypothesis though compatible with the other two. The third hypothesis appears, however, to be the only one compatible with the results of Stockdale et al. already described (Stockdale et al., 1970). Aldridge and Rose (Aldridge and Rose, 1969) have suggested that the site of binding of triethyltin involves two histidine residues. Studies on

triethyltin binding to rat hemoglobin have shown that two molecules are bound per hemoglobin tetramer and that each of the binding sites involves two histidine residues. Rose (Rose, 1969) and Rose and Lock (Rose and Lock, 1970) have similarly deduced from studies of the binding of triethyltin with a protein fraction from guinea pig liver, that the binding site for triethyltin involves histidine residues.

Trialkyltins, unlike oligomycin, also inhibit photophosphorylation. Kahn (Kahn, 1968) has shown that chlorotri-n-butyltin is a specific inhibitor of photophosphorylation in Euglena chloroplasts and that no effects on electron transport can be observed. In a manner analogous to the effect of oligomycin on sub-mitochondrial particles (Lee and Ernster, 1968), low concentrations of chlorotributyltin were found to stimulate both photophosphorylation and the light dependent 'pH rise' in chloroplasts which were deficient in coupling factors.

'1799'

Little is known about this uncoupler other than what has been reported by Heytler (Heytler, 1970). It is a potent uncoupler not only of mammalian mitochondria but also of yeast mitochondria (Groot et al., 1971).

chloro CCP and CCP Derivatives

These substances uncouple not only oxidative phosphorylation (Heytler and Pritchard, 1962; Heytler, 1963) but also cyclic photophosphorylation in spinach chloroplasts (Heytler, 1963; Avron and Shavit, 1965). chloro CCP has been shown to uncouple mitochondria, to activate the ATPase activity of intact mitochondria and to block the swelling of mitochondria induced by ADP or thyroxine. It also inhibits the $^{32}P_i$ - ATP exchange reaction of rat liver mitochondria. In all these respects, therefore, chloro CCP is acting at least superficially, analogously to DNP. Unlike DNP, however, it does not stimulate the activity of purified ATPase preparations. In long-term experiments chloro CCP has been shown not only to uncouple succinate oxidation but also to cause inhibition of the rate of oxidation. The latter but not the former effect was prevented by the addition of cysteine sulfinic acid, and has been attributed to its effect on the accumulation of oxaloacetate, an inhibitor of succinic dehydrogenase (Goldsby and Heytler, 1963).

chloro CCP has been shown to cause a dissipation of the proton gradient across the membranes in isolated chloroplasts and chromatophores (Jackson et al., 1968), phospholipid bilayers (Hopfer et al., 1968) and mitochondria (Mitchell, 1968).

Various detailed mechanistic theories have been proposed to account for the way in which this proton gradient is dissipated, the interpretations drawing heavily on the experimenter's commitment to either the chemiosmotic or chemical hypotheses of oxidative phosphorylation.

Mitchell has proposed that uncouplers such as chloro CCP move across the membrane in conjunction with a proton, thus collapsing the pH gradient, and then move back outwards as the anion, thus collapsing the charge difference (Mitchell, 1968). Van Dam has proposed, on the other hand, that the initial step is an active inward pump of the uncoupler anion in exchange for a hydroxyl ion, followed by a passive diffusion of the undissociated acid (Van Dam and Slater, 1967 ; Kraayenhof and Van Dam, 1969). Weinbach and Garbus have suggested that uncouplers like chloro CCP interact more directly with the enzyme or protein actually concerned in energy conservation, leading to conformational changes resulting in the uncoupling phenomena (Weinbach and Garbus, 1969). Margolis et al. (Margolis et al., 1967) have hypothesised that uncouplers such as the CCP derivatives act by titrating and binding only against actively phosphorylating sites in the mitochondria. They suggest that these uncoupling substances, like chloro CCP, are highly mobile within the mitochondrial membrane and in respect of this 'sub-stoichiometry', CCP derivatives resemble TTFB. (For further discussion of the sub-stoichiometry, see TTFB section). Kurup and Sanadi (Kurup and Sanadi, 1968) concluded that FCCP must bind to a component in the energised state and found that 1 mole of the uncoupler was bound per Site II and III whilst 14 moles were bound per Site I. The latter observation has been interpreted as showing there are either 14 FCCP binding components per Site I or each respiratory chain or that the binding site has much lower affinity for FCCP than Sites II and III.

As far as yeast is concerned, chloro CCP has been shown to uncouple the respiration of yeast mitochondria as well as mammalian mitochondria (Groot et al., 1971). It is also effective in uncoupling the respiration of intact yeast cells (Kovác et al., 1970) and, moreover, inhibits the respiratory adaptation of anaerobic yeast (Kovác et al., 1968b).

TTFB

Studies on the biological activity of substituted benzimidazole derivatives have shown that they inhibit the growth of many organisms including S.cerevisiae, bacteria

and viruses (Wooley, 1944; Bishop et al., 1964; Tamm et al., 1953). Buchel et al. (Buchel et al., 1965a) and Beechey (Beechey, 1966) have reported that some substituted benzimidazole derivatives are highly effective uncouplers of mitochondrial oxidative phosphorylation and found that the derivatives were progressively better uncouplers as both their N - H acidity and the degree of dissociation of the compound at pH 7.4 increased. This has been interpreted as demonstrating that the active uncoupler is actually the substituted benzimidazole anion. The most effective of the derivatives they tested was TTFB (4,5,6,7-tetrachloro-2-trifluoromethyl benzimidazole) which was found to uncouple at concentrations as low as 3×10^{-8} M. Beechey (Beechey, 1966) found that TTFB apparently acted identically to the classical uncoupler DNP except, of course, that it was very much more potent. Liberman and Topaly have shown that, like DNP, TTFB is a proton conductor in bimolecular phospholipid membranes (Liberman and Topaly, 1968). Calculations of the stoichiometry of uncoupler binding to mitochondria have shown, however, that TTFB differs from DNP in that it is an effective uncoupler even when present in molecular amounts considerably lower than the total number of potential coupling sites (Margolis et al., 1967). In exhibiting this 'sub-stoichiometry' TTFB behaves like the substituted carbonyl cyanide phenyl-hydrazone derivatives. Margolis et al. (Margolis et al., 1967) have concluded that compounds such as FCCP showing such behaviour uncouple by titrating and discharging 'high energy' intermediates, binding only to active coupling sites. Similar findings have been reported by Kurup and Sanadi (Kurup and Sanadi, 1968).

Under conditions where oxidation rates were maximal, a stoichiometry of one TTFB binding component per Site II and III phosphorylation site was found. These results have been taken to favour the chemical rather than the chemiosmotic hypothesis though Nicholls and Wenner (Nicholls and Wenner, 1970) have disputed whether the mode of action of the two different classes of uncouplers is really qualitatively different, taking S13 as an example of an uncoupler showing 'sub-stoichiometry.' It should perhaps be mentioned in this regard that Wilson (Wilson, 1969) has stated that there is a strict stoichiometry of S13 to respiratory assemblies which is independent of the rate of electron flux or 'high energy' intermediate generation. This did not hold, however, when the effects of S13 on succinate oxidation in the presence of malonate was examined.

Substituted benzimidazole derivatives have also been shown to inhibit photophosphorylation (Buchel et al., 1965b). This effect is due to the uncoupling of photophosphorylation from electron flow and there is no inhibition of photosynthetic reduction of NADP itself when an artificial electron donor is used (diaminodurol / ascorbate). Benzimidazole itself has been shown to have a number of effects on enzymic synthesis and respiratory adaptation of yeast cells (Sels, 1969).

DNP and octyl DNP

The nitrophenols are the classical uncouplers of cellular respiration. Lardy et al. first suggested in 1945, after DNP was reported to stimulate the ATPase activity of minced rat liver muscle, that this drug acted either by catalysing the breakdown of an intermediate in oxidative phosphorylation or by dissociating oxidation from phosphorylation (Lardy and Elvehjen, 1945). Despite continuing discussion on the actual mechanism of uncoupling occurring with substances such as DNP (see reviews by Lardy and Ferguson, 1969; Van Dam and Meyer, 1971), the actual characteristics of DNP uncoupling are well documented. DNP has been shown to stimulate the respiration of isolated coupled mitochondria under assay conditions where the medium is deficient in phosphate acceptor, to abolish the ATP synthesis normally coupled to mitochondrial respiration with the exception of the substrate-linked phosphorylation involved in the oxidation step of α ketoglutarate → succinate, to activate mitochondrial ATPase activity and to inhibit the ^{32}Pi - ATP, ADP - ATP exchange reactions as well as the exchange of oxygen atoms between water and Pi, and water and ATP (Slater, 1963). DNP also causes an inhibition of respiration when both low and high concentrations of the DNP are used. This inhibition is masked, however, when low concentrations of DNP are used by the stimulation of respiration caused by its uncoupling activity (Van Dam, 1967). Van Dam and Slater have suggested a unified hypothesis for explaining the uncoupler and inhibitor activities of uncouplers such as DNP (Van Dam and Slater, 1967) involving an energy-linked non-specific anion carrier for which the uncoupler anions also show affinity. Mitchell (Mitchell, 1961) has suggested that DNP can act as an ion conductor, a suggestion supported by the results of Hopfer and coworkers (Hopfer et al., 1968), Blewelaski and Lehninger (Blewelaski and Lehninger, 1966) and Liberman and Topaly (Liberman and Topaly, 1968) who have shown that DNP exhibits a high specificity for proton transport.

The relative effectiveness of the different nitrophenols such as dinitrophenol and octyl dinitrophenol as uncouplers has been shown to depend on both the pK of the compound and its lipid solubility. The concentration of the compound needed for maximum uncoupling has been found to fall with increasing lipid solubility of the compound (Slater, 1963). DNP is not an effective uncoupler of photophosphorylation (Losada and Arnon, 1963) though it can act as a weak uncoupler when added to chloroplasts incubated within a narrow pH zone at high concentrations (Neumann and Jagendorf, 1964). DNP at high concentrations more generally acts as an inhibitor of photophosphorylation than as an uncoupler (Losada and Arnon, 1963 ; Neumann and Jagendorf, 1964). DNP is known not only to stimulate respiration in intact yeast cells but also to inhibit energy-requiring processes in yeast cells under anaerobic conditions (Spiegelman, 1947 ; Reiner and Spiegelman, 1947 ; Kovác and Istenova, 1964). Jarett and Hendler (Jarett and Hendler, 1967) have shown that DNP is a potent inhibitor of both RNA and protein synthesis in anaerobic yeast cells. Similar findings of inhibition of energy-requiring processes by DNP in bacteria (Fowler, 1951) and animal cells (Jarett and Kipnis, 1967) have also been made. To account for all these reports of inhibition by DNP under anaerobic conditions when oxidative phosphorylation is not functioning and ATP is provided only by glycolysis, it has been assumed that a hypothetical high energy intermediate of phosphorylation equilibrates with glycolytically formed ATP and can be used as a direct energy source for some cellular processes, including protein synthesis (Slater, 1953). Alternative explanations for these observations are that uncouplers such as DNP either cause disturbances in the appropriate redox state of the cell or have a more general effect on membrane functions. It should be noted that higher concentrations of uncoupling agents are generally necessary to inhibit synthesis of the respiratory chain during respiratory adaptation or of the maltose fermenting system under anaerobic conditions than are needed to uncouple mitochondrial function.

Characteristics of S.cerevisiae

S.cerevisiae is a uni-cellular eucaryote which can exist in both the haploid and diploid states, diploidisation of haploid cells being brought about by the fusion of two haploid cells of opposite mating type 'a' and 'α'. In both the haploid and diploid states the cells are able to multiply asexually by budding, this resulting in

clusters of 2 - 4 cells often being linked to each other. The diploid cell can be induced to sporulate, when a classical meiotic division ensues during which the diploid cell nucleus undergoes two successive divisions, only one of which is a reduction division, to form four haploid nuclei which give rise to four spores. These are retained together with an ascus. Breakage of the ascus and release of the spores enables the haplophase of the cell cycle to be resumed.

S.cerevisiae is a facultative anaerobe, able to grow anaerobically in the presence of fermentable substrate. A wide variety of both fermentable and non-fermentable carbon substrates can be utilised by S.cerevisiae, though the latter obviously can be utilised only under aerobic conditions. Thus, mutants which lack a complete respiratory chain are still viable if grown with glucose or other fermentable substances as the carbon source. Mitochondrial formation in S.cerevisiae is repressed not only under anaerobic conditions but also under conditions of catabolite repression caused (indirectly) by high glucose concentrations. In both cases the formation of a large number of mitochondrial enzymes and other components is inhibited (Linnane and Haslam, 1970). The mitochondria of S.cerevisiae are generally rod-shaped or spherical bodies with a diameter of about 0.3 - 1.0 μ and a length of 3 μ. Their structure is generally similar to that of mitochondria extracted from higher plant and animal cells. Mitochondria from yeast cells are characterised by their high lipid content which can account for 25.4% of the mitochondrial dry mass (Matile et al., 1969). Various claims that mitochondria extracted from S.cerevisiae or S.carlsbergensis possess only two phosphorylation sites have been made (Vitols et al, 1961), the first site, Site 1, being absent. This has, however, been disputed by other workers (Chance, 1959; Ghosh and Bhattacharyya, 1970; Schuurmans-Steckhoven, 1966) whilst Onishi has found that the Site I, though not the 'g = 1.94' signal of iron sulphur proteins in the NADH dehydrogenase region, can be induced by aeration of non-growing cells prior to mitochondrial extraction (Onishi, 1970).

Use of S.cerevisiae in this investigation has several advantages. Firstly, its genetics is, for a eucaryote, reasonably well understood and many auxotrophic marker genes for different biochemical pathways are available. Secondly, a great deal of progress in understanding its mitochondrial genetics has been made recently. Thirdly, being a facultative aerobe, it is possible to obtain cells carrying mutations

affecting the mitochondrion, which would not be recovered in an obligate aerobe due to their lethal effect. Fourthly, a reasonable amount of information about the biochemical characteristics of the mitochondria of S.cerevisiae has been accumulated although this is obviously less than is known about mammalian mitochondrial systems.

The Cytoplasmic Genetics of S.cerevisiae

Various reviews of the whole or part of general yeast genetics (Mortimer and Hawthorne, 1969; Hartwell, 1970; Fincham, 1970; Fogel and Mortimer, 1971; Sherman and Stewart, 1971) and cytoplasmic yeast genetics (Roodyn and Wilkie, 1968; Coen et al., 1970; Linnane and Haslam, 1970; Bolotin et al., 1971; Preer, 1971) have only recently been published. In view of this, only the major aspects of the subject and areas where further clarification would be of value are considered below.

Killer Character

Bevan and Makower (1963) have suggested that there are three types of yeast cell with respect to this character - killers, sensitives and neutrals. The killer phenotype is determined by a cytoplasmic factor 'k' which, however, is only maintained in the cytoplasm when a nuclear gene 'm' is in the dominant allelic form. In the presence of the allelic form 'm' of this gene, the factor 'k' disappears. Sensitive cells lack the cytoplasmic factor 'k' but may have either of the allelic forms of the nuclear gene 'm' present. Neutrals have a different cytoplasmic factor 'n' conferring their phenotype but this factor is again dependent on the dominant allelic form of the gene 'm' for its maintenance. Crosses of killers x sensitives give rise to killer diploids, these hybrids giving tetrad ratios of 4:0 killer if the sensitive strain carried 'M' whilst ratios 2:2 killer:sensitive are found if the sensitive haploids carry the allelic form 'm' of the nuclear gene. The same pattern of inheritance is seen in crosses of neutrals x sensitives, whilst crosses of killers x neutrals give rise to 'weak killers.' Diploids from the latter crosses give rise on mitotic division during vegative propagation to variant clones which are either strong killer or neutral cells, whilst meiotic division of these 'weak killers' results in highly abnormal tetrad ratios (Somers and Bevan, 1969). The agent responsible for the killing effect of the killer strains has been shown to be a protein (Bevan and Somers, 1969). Neither the 'k' nor 'n' factors appear synonomous with the rho factor of the mitochondrion.

The Petite Mutation

The most widely investigated cytoplasmic mutation in S.cerevisiae is the petite mutation which has been extensively investigated by Ephrussi and his collaborators. Phenotypically, the most marked characteristic of petite mutants is that on plating onto glucose medium, such mutants form only small colonies unlike the wild type cells of 'grandes' which form large colonies. Petites fail to grow on non-fermentable medium and do not show functional electron transport when colonies are examined after growth on YEPG medium with the TTC overlay technique (Roodyn and Wilkie, 1968; Nagai, 1959). Two types of petite may be distinguished; the cytoplasmic and the nuclear or genic petites, both displaying the phenotypic characteristics described above. By far the majority of petites isolated normally are cytoplasmic petites as these occur spontaneously at the very high frequencies of 1×10^{-1} - 1×10^{-3} as opposed to the nuclear petite mutational rate of around 1×10^{-6} - 1×10^{-7} (Roodyn and Wilkie, 1968).

The cytoplasmic petites are characterised by a loss of cytochromes $a + a_3$, b and c_1 and certain dehydrogenases, whilst, in addition, marked changes in the levels of the $L^{(+)}$ and $D^{(-)}$ lactate dehydrogenase activity of the petite cell is observed (Roodyn and Wilkie, 1966). Further consideration of the changes in enzyme activity in the petite is given by Sherman and Slonimski (Sherman and Slonimski, 1964a) and Roodyn and Wilkie (Roodyn and Wilkie, 1966). Electron microscopic examination of the cytoplasmic petites has shown a loss of mitochondrial structure and definition in the majority of cases involving a loss of the inner mitochondrial cristal membranes (Yotsuyanagi, 1962; Avers et al., 1965; Smith et al., 1969).

One of the characteristics of the cytoplasmic petite mutant is its stability. Thus, despite the high frequency of petite formation, revertants from petite to wild type have not been found (Roodyn and Wilkie, 1968). This has led to the assumption that cytoplasmic petites are caused by the deletion of a genetic locus (loci) rather than by its local structural alteration. Genetic analysis has shown that the factor concerned is cytoplasmically inherited, some petites showing mitotic segregation of both petite and wild type diploid cells from crosses of a petite by a grande, whilst all wild type diploids from such crosses on sporulation give only wild type haploids, rather than the 2:2 ratio expected for a gene showing Mendelian inheritance. The cytoplasmic factor concerned has been termed the

rho (ρ) particle (Sherman and Slonimski, 1964b) and cytoplasmic petites are referred to as ρ^- strains. The finding that the mitochondrial DNA extracted from petites is either absent or grossly changed in its base composition and buoyant density, has led to the correlation of the rho factor with mitochondrial DNA (Corneo et al., 1966; Mounolou et al., 1966; Michaelis et al., 1971; Moustacchi and Williamson, 1966). It was originally thought that the change in base composition of the mDNA which can lead to this DNA being almost an A :T homopolymer was due solely to deletion of particular base sequences of the mDNA. As Fukuhara, however, has recently presented evidence that the changes involved result in amplification of some of the regions of the mitochondrial genome not deleted, this is probably oversimplistic (Fukuhara, private communication). Cytoplasmic petites do not constitute a homogeneous class of mutants and clear differences between petite mutants are evident even though all possess superficially the same phenotype in being respiratory deficient. Petites are known to differ in

(a) the amount, base composition and buoyant density of their mDNA

(b) the degree of retention of genetic information as measured by their retention or otherwise of antibiotic resistant mitochondrial genes (see below)

(c) their degree of suppressiveness (see below)

When haploid cytoplasmic petites are crossed by ρ^+ haploids, some petites give only wild type diploid progeny when account is taken of the spontaneous petite frequency. These petites are known as neutral petites and they have been shown to be completely lacking in mDNA (Nagley and Linnane, 1970; Goldring et al., 1970; Michaelis et al., 1971). Other petites in identical crosses give rise to diploid progeny containing up to 99% petites. Such haploid petites which give, on crossing to grande haploids petite progeny in numbers above the spontaneous background, are known as suppressive petites (Ephrussi et al., 1955; Ephrussi et al., 1966). The degree of suppressiveness of a petite appears to depend to some extent on its manner of isolation. Petites induced by high levels of ethidium bromide tend to almost all of low or neutral suppressiveness (Linnane and Haslam, 1970; Saunders et al., 1971). Suppressiveness in petites is an unstable characteristic, strains changing their degree of suppressiveness during continued vegetative sub-

cloning. In general, however, a mother daughter relationship in the degree of suppressiveness is maintained, with, moreover, both neutral and highly suppressive petites tending to maintain their suppressiveness without change, instability being confined mainly to petites exhibiting intermediate suppressiveness (Saunders et al., 1970; Ephrussi et al., 1966). Attempts to relate the phenomenon of suppressiveness to the degree of retention of mitochondrial genetic information by petites have not been altogether successful (Saunders et al., 1970) even though suppressiveness appears to be cytoplasmically inherited (Ephrussi et al., 1955).

A number of authors have studied the relationship of suppressiveness to the changes in buoyant density in mDNA apparent in petites. Although the results are equivocal, there is a general movement with increasing suppressiveness to greater simplicity in the petite mDNA (Mehrotra and Mahler, 1968; Carnevali et al., 1966). No case of recombination between different petites to give a grande cell has yet been reported despite extensive efforts. The advent of petites carrying marked sequences by virtue of the antibiotic resistance sequences they carry, may, however, allow resolution of whether such lack of recombination is due to overlapping deletions in the petites tested or an effect of the petite mutation on the cytoplasmic recombination process per se.

Antibiotic Resistant Mutants

Cytoplasmic mutants resistant to a variety of antibiotics including chloramphenicol, spiramycin, erythromycin, mikamycin, paromomycin and oligomycin have been isolated (Wilkie et al., 1967; Thomas and Wilkie, 1968a; Coen et al., 1970; Thomas and Wilkie, 1968b; Bunn et al., 1970; Stuart, 1970; Linnane et al., 1968). These mutants exhibit cytoplasmic inheritance in that they show mitotic segregation in crosses of the type $Ant^{R}_{\rho}{}^{+}$ x $Ant^{S}_{\rho}{}^{+}$, and in that sporulation of diploids from such crosses gives rise to asci containing either 0:4 or 4:0 resistance : sensitive ascospores, depending on whether the diploid sporulated was sensitive or resistant to the antibiotic. Experiments in which $Ant^{R}_{\rho}{}^{+}$ x $Ant^{S}_{\rho}{}^{-}$ crosses failed to show mitotic segregation and other experiments demonstrating that the conversion of an $Ant^{R}_{\rho}{}^{+}$ strain to a ρ^{-} strain was accompanied by a loss of the resistance determinant, have allowed the cytoplasmic determinants to be localised on the mDNA (Coen et al., 1970; Linnane et al., 1968; Gingold et al., 1969). The isolation of these antibiotic resistant mutants has allowed studies on the process of

mitochondrial recombination. That such recombination occurs is evident from the finding that crosses of $Ant^{S}\ \rho^{+}$ haploids by $Ant^{R}\ \rho^{-}$ cells can give rise to both Ant^{R} and $Ant^{S}\ \rho^{+}$ diploids (Coen et al., 1970). Thomas and Wilkie first reported recombination occurring between different drug resistant mutants using strains carrying the erythromycin, spiramycin and paromomycin resistance determinants (Thomas and Wilkie, 1968b).

Slonimski and coworkers have studied, using these antibiotic resistant mutants, two related processes. Firstly, the distribution and segregation of particular cytoplasmic markers in the diploid progeny of crosses of the type $Ant_1^{R}\ \rho^{+} \times Ant_1^{S}\ \rho^{+}$, and secondly the mitotic recombination processes occurring when two and three factor crosses of the type Ant_1^{R}, $Ant_2^{S}\ \rho^{+} \times Ant_1^{S}$, $Ant_2^{R}\ \rho^{+}$ are made (Coen et al., 1970; Bolotin et al., 1971). The segregation patterns observable for particular markers in single factor crosses when analysed on a clonal basis have been shown to be complex and to differ from strain to strain, with, in at least one case, possibly two different genic transfer mechanisms involved in the transmission process (Coen et al., 1970). Analysis of the two and three factor crosses apart from revealing the kinetics and the clonal segregation patterns, has shown that the recombination frequencies are influenced by the presence of a mitochondrial 'sex' factor (Bolotin et al., 1971). This factor, which was originally intimated to be identical to the cellular mating type gene which is a nuclear gene, has since been shown to be quite distinct from this and to show cytoplasmic inheritance (Bolotin et al., 1971). This mitochondrial 'sex' factor which has been named ω exists in three naturally occurring states, ω^{+}, ω^{-} and ω^{o} (Slonimski, private communication). Of the strains analysed, the majority of grandes are ω^{+} whilst the rest, the minority, are ω^{-}. ω^{+} strains carrying markers for the C and E (chloramphenicol and erythromycin) loci when mated with an ω^{+} tester (arbitrarily designated as such) show, amongst the recombinants for the C - E markers, a lack of polarity, i.e. the proportion of the reciprocal recombinants are equal or

$$\frac{C_1E_2}{C_2E_1} \simeq 1 \quad *$$

* In this passage, one of the crossing strains has been called 1 and the other 2. Thus, C_1 is the chloramphenicol resistant gene derived from strain 1 and C_2 from strain 2.

Similar results are obtained in crosses of ω^- x ω^- strains and both these types of crosses are referred to as homosexual crosses. Crosses of ω^+ strains by ω^- strains are designated heterosexual crosses and are characterised by the proportion of the reciprocal recombinants being greater than one, i.e.

$$\frac{C_1E_2}{C_2E_1} > 1$$

This has been termed polarity, the numerical value for the polarity allowing estimation of the relative position of the genetic markers (Bolotin et al., 1971) as this polarity is apparently dependent on the distance of the markers from an apparent fixed point (ω ?). Thus, markers close to this point show high polarity, e.g. chloramphenicol resistance whilst the determinant for oligomycin resistance shows little polarity. Polarity is, of course, also evident in heterosexual single factor crosses, i.e. the proportion of $C_1/C_2 > 1$, but the transmission value obtained for single factor crosses appears more prone to fluctuation due to the nuclear constitution of the strain than do the recombinant frequencies (Coen, private communication). All the recombinants for mitochondrial genes from heterosexual crosses have been shown to be ω^+.

The third naturally occurring form of ω is ω^0, which has been found only in petite strains and is presumed due to a loss of the ω function. Petites which are ω^0, even where obtained from ω^+ grandes give, in crosses by ω^- grandes, only ω^- progeny (Slonimski, private communication). Mutants of the ω function in grande cells have also been obtained (Dijon, private communication) and these have been designated ω^n. Such mutants were obtained starting from an ω^+ strain. They are characterised by the fact that in crosses by both ω^+ and ω^- tester strains, no polarity is apparent. The strains carrying this ω allele, in other words, appear to possess hermaphroiditism in respect of their mitochondrial sex.

The use of the phenomena of polarity, the estimation of recombination frequencies between markers, and the effects of u.v. on the mating recombination process have all been used by Slonimski and collaborators to map the position of the determinants responsible for the antibiotic resistance of the various cytoplasmic mutants (Bolotin et al., 1971). A further approach used by this group has been to use the ability of some petites to retain genetic information as exemplified by the presence of mitochondrial genes determining resistance or sensitivity, to establish

a deletion map of the mitochondrial genome (Slonimski, private communication).

CHAPTER 2

MATERIALS AND METHODS

Materials

Phenotypic and Genetic Sections Chapters 3, 4, 5, 6 and 7

Acriflavine Neutral was purchased from the Sigma Chemical Company.

Antimycin A was bought from the Sigma Chemical Company.

Aurovertin was a kind gift from Dr. H. A. Lardy and Dr. R. B. Beechey.

Chloro CCP was purchased from Calbiochem.

Cycloheximide was purchased from the Sigma Chemical Company.

Chloramphenicol was either purchased from the Sigma Chemical Company or was donated by Parke Davis and Company.

DCCD was bought from British Drug Houses Limited.

DNP was obtained from British Drug Houses Limited.

Erythromycin was a kind gift of the Eli Lilly Company Limited. It was also purchased from the Sigma Chemical Company.

Ethidium Bromide was kindly donated by Boots Pure Drugs Company Limited.

Mikamycin was purchased from Calbiochem.

Nystatin was kindly given by E. R. Squibb and Son Limited.

Oligomycin was purchased from the Sigma Chemical Company.

Rutamycin was generously donated by the Eli Lilly Company Limited.

Snail enzyme was obtained from L'Industrie Biologique Francaise S.A., Gennevilliers, France.

Spiramycin was a generous gift of May and Baker Limited.

Triethyltin Sulphate was a kind gift of Dr. W. N. Aldridge.

Triphenyl-tetrazolium Chloride was obtained from British Drug Houses Limited.

Trizmabase was purchased from the Sigma Chemical Company.

TTFB was generously given by Dr. R. B. Beechey.

'1799' was a gift from Dr. Heytler.

Peptone, Ionagar and Agar No. 3 were obtained from Oxoid Limited and

Yeast Extract from Difco Laboratories.

All other reagents were obtained from either Hopkins and Williams Limited or British Drug Houses Limited and, except for those used in media, were of 'Analar' grade purity.

Yeast Strains

The haploid strains used routinely in this investigation for the isolation of the mutants and their characterisation were :

D22 a ad_2^- arg^+ met^+ ρ^+ ω^+ $C^S E_r^S OL^S Sp^S Cy^S CP^S$

D6 α ad_2^+ arg^- met^- ρ^+ ω^+ $C^S E_r^S OL^S Sp^S Cy^S CP^S$

Both these strains were the kind gift of Dr. D. Wilkie. The following additional strains were used during the mapping and 'sexing' studies in the laboratory of Professor P. P. Slonimski :*

IL126-1C a ur^- ρ^+ ω^- C^R_{321} E^R_{221} O^S

IL126-3A α his^- ρ^+ ω^- C^R_{321} E^R_{221} O^S

IL8-8C α his^- try^- ρ^+ ω^+ C^R_{321} E^R_{514} O^S

IL8-8D a ur^- ρ^+ ω^+ C^R_{321} E^R_{514} O^S

IL778-1A a his^- ur^- ρ^+ ω^- C^R_{321} E^S O^R_I

IL781-1A a ur^- ρ^+ ω^+ C^R_{321} E^S O^R_I

IL779-1B a his^- ur^- ρ^+ ω^+ $C^S E^R_{221}$ O^R_I

IL781-5B a ur^- ρ^+ ω^+ C^R_{321} E^S O^R_I

IL836-4B α ur^- ρ^+ ω^+ $C^S E^S$ O^R_I

Solid Growth Medium

(a) Non-Selective

The growth media used routinely for the genetic studies were :

* Nomenclature : $O^R_{E^R_{S^R}}$ (Slonimski) = $OL^R_{E_r^R Sp^R}$ (Avner)

YEPG	1% yeast extract, 2% peptone, 2% glucose, 2% Agar No. 3
YEPGly	1% yeast extract, 2% peptone, 4% glycerol, 2% Agar No. 3
YEP EtOH	1% yeast extract, 2% peptone, 4% EtOH, 2% Agar No. 3
YEP PDM (petite determinant medium)	1% yeast extract, 2% peptone, 4% glycerol, 0.1% glucose, 2% Agar No. 3
YEPGA	1% yeast extract, 2% peptone, 2% glucose, adenine 20 mg/litre, 2% Agar No. 3

The following defined media were also used :

MMGlu	Wickerhams Minimal Medium (Wickerham, 1946) + 2% glucose + 1.5% Ionagar
MMGly	Wickerhams Minimal Medium + 4% glycerol + 1.5% Ionagar
MM EtOH	Wickerhams Minimal Medium + 4% EtOH + 1.5% Ionagar

When these media were supplemented with adenine, arginine and methionine, the following concentrations were used : 100 mg/litre, 10 mg/litre and 10 mg/litre respectively.

All these media were routinely used unbuffered, their final pH being around 5.8.

Sporulation Media

Two media were used :

(1) McClary's (McClary et al., 1959)

0.25% yeast extract, 0.1% glucose, 0.98% K^+ acetate (anhydrous), 1.5% Ionagar - adjusted to pH 6.9 with KOH.

(2) Kleyn's (Kleyn, 1954)

0.25% tryptone, 0.6% glucose, 0.062% NaCl, 0.5% Na^+ acetate, $3H_2O$, 1.5% Ionagar.

Presporulation Medium

5% glucose, 5% nutrient broth, 1% yeast extract (Fowell, 1969).

Tetrad Dissection Medium

2% Ionagar in 0.1 M phosphate buffer pH 7.0.

(b) Selective

Drug plates were normally made by adding an ethanolic solution of the drug to autoclaved media which had been cooled to 45 - 50°C after autoclaving. The only exceptions to this practice were with chloramphenicol which was added as a solid prior to autoclaving, and $CoSO_4$ which was added as a solid after autoclaving.

Ethidium bromide and acriflavine were added to solid media as aqueous solutions after the autoclaving of the rest of the medium. All drugs were normally added to the unbuffered media outlined above, the only exceptions being with DNP and DCCD drug plates and here the procedure is fully outlined in the relevant section of the thesis.

Unless otherwise stated, the specific action of the drug on mitochondrial metabolism was tested using YEPGly rather than YEP EtOH media and its non-specific mode on general cell metabolism using YEPG medium. Only in the specific case of DCCD resistance and for certain experiments with diploid strains where it was necessary to avoid haploid contamination was drug resistance tested using the defined medium MMGly, MM EtOH and MMGlu.

The experiments carried out in collaboration with Slonimski's group were all performed using buffered media as outlined by Coen et al. (Coen et al., 1970) with the addition that buffered YEPGly oligomycin plates (2.5 γ/ml) were also used.

Liquid Growth Medium - Non-Selective and Selective

The liquid growth medium used was identical to those used for plating work, agar, however, being omitted from such media. In the case of YEP EtOH and MM EtOH however, modified EtOH concentrations of 0.5% and 1% were normally used rather than the 4% added to the solid medium as it was found that cells on the latter medium had a much slower growth rate, presumably due to ethanol inhibition. Normally 100 ml of liquid medium was used in 500 ml conical flasks and 50 ml in 250 ml conical flasks. Drug additions, where made, were the same as for solid media - but the additions were made to media which had been cooled to room temperature. Antifoam, where added to liquid medium, was MS Silicone Antifoam A and was added at a concentration of 1 ml/litre prior to autoclaving, or autoclaved separately and added afterwards. All media which were used were made up in distilled water.

Sterilisation

Sterilising of media was generally carried out by autoclaving for 15 minutes at 15 lbs/p.s.i. pressure. Where quantities larger than 1 litre were sterilised, the sterilisation was carried out for 30 minutes at 15 lbs/p.s.i. pressure.

Solutions not suitable for autoclaving were Millipore filtered using pre-autoclaved apparatus with GS filters. All glassware was sterilised by dry heat at 200°C for 4 - 6 hours.

Strain Purification and Preservation

Strain purification was routinely undertaken every three months and the following markers were checked :

D22 and D22 OL^R strains $\frac{ad_2^-}{ad_2^+}$ $\frac{OL^S}{OL^R}$ $\frac{\rho^-}{\rho^+}$

D6 and D6 OL^R strains $\frac{arg^-}{arg^+}$ $\frac{met^-}{met^+}$ $\frac{OL^S}{OL^R}$ $\frac{\rho^-}{\rho^+}$

D22 Er^R strains $\frac{ad_2^-}{ad_2^+}$ $\frac{Er^S}{Er^R}$ $\frac{OL^S}{OL^R}$ $\frac{\rho^-}{\rho^+}$

D22 CP^R strains $\frac{ad_2^-}{ad_2^+}$ $\frac{CP^S}{CP^R}$ $\frac{OL^S}{OL^R}$

D6 CP^R strains $\frac{arg^-}{arg^+}$ $\frac{met^-}{met^+}$ $\frac{CP^S}{CP^R}$ $\frac{OL^S}{OL^R}$

Strains were stocked on YEPG slopes and stored in the cold room at $0^\circ C$.

Procedure for u.v. Mutagenesis

Cells grown overnight at $30^\circ C$ in liquid YEPG medium were spun down using a bench centrifuge and the cells washed with sterile 0.1 M phosphate buffer, pH 7.3, before being resuspended at a final concentration of 2×10^7 cells/ml in the same buffer. 60 ml of this suspension was placed in a sterile dish (diameter 8 cm) fitted with a magnetic stirrer and placed in a sterile u.v. cabinet fitted with a filtered air supply. U.v. irradiation was undertaken for the times mentioned in the text. The u.v. source was a Sylvania u.v. tube (type G8T5) placed 10 centimetres from the surface of the irradiated cell suspension. Following irradiation, cells were plated out immediately and the plates incubated at $30^\circ C$ for the times mentioned in the text. Precautions were taken to prevent photo-repair of u.v. damage during the post-irradiation period.

Procedure for Nitrous Acid Mutagenesis

Cells grown overnight at 30°C in liquid YEPG medium were spun down, washed once with saline solution (0.1% KH_2PO_4, 0.05% $MgSO_4$, 0.01% NaCl and 0.01% $CaCl_2$, adjusted to pH 7.0), and then either with or without prior starvation in this saline solution, 2×10^7 cells were spun down and resuspended in 9 ml of 0.2M acetate buffer, pH 4.0. To this was added 1 ml of freshly made sodium nitrite solution in distilled water to give a final concentration of 0.3M sodium nitrite. The nitrous acid treatment, thus generated, was stopped by withdrawing samples and diluting them at least fifty-fold with 0.1M phosphate buffer, pH 7.3. The nitrous acid treatment was carried out at 30°C (Sherman and Slonimski, 1964b).

Drug Resistance Assays

Drug resistance of the strains was normally tested by dropping out cell suspensions onto the drug plates and the control medium minus drugs using a multi-prong inoculator capable of transferring simultaneously 23 strains to the plating medium.

Mating Procedure

Diploids were obtained using the mass mating, and prototrophic selection technique of Pomper and Burkholder (Pomper and Burkholder, 1949). Haploids were precultured for 24 - 64 hours in liquid or solid YEPG or YEPGly prior to mating. In the case of the drug resistant mutants, the preculturing was generally carried out in YEPGly + the drug to avoid sensitive revertants. Where the preculture was liquid (10 ml), the cells were collected by centrifugation and washed once with sterile distilled H_2O before resuspending in a small amount of sterile distilled H_2O (1 - 2 ml) and dropping onto the MMGlu plates. With solid precultures, a scrape was suspended into 1 - 2 ml of sterile H_2O, mixed thoroughly and dropped out onto the MMGlu medium. Most of the crosses described in this thesis were made with liquid precultures.

Following the dropping out onto MMGlu and mixing of compatible haploids (α and a mating types) the plates were incubated at 30°C for 3 - 4 days when the diploids able to grow in the absence of arginine, methionine and adenine were available for analysis.

Analysis of Oligomycin Resistance
Transmission by Quantitative Aliquot Plating

After the diploids had been obtained as described above, some 10 - 20 generations after the zygotic fusions, they were removed from the mating plate and resuspended in sterile H_2O. After suitable dilution, a range of cell concentrations was applied directly to MMGly plates with and without oligomycin. Dilutions were adjusted so that the interval between successive dilutions was approximately equivalent to a thirty-fold drop in cell density, and the dilutions were so arranged that 0.2 ml of the most dilute sample used in the absence of oligomycin gave about 100 - 150 viable cells per plate. The plates were incubated at 30°C for 5 - 7 days before scoring. A similar procedure was followed when erythromycin resistance transmission was assayed.

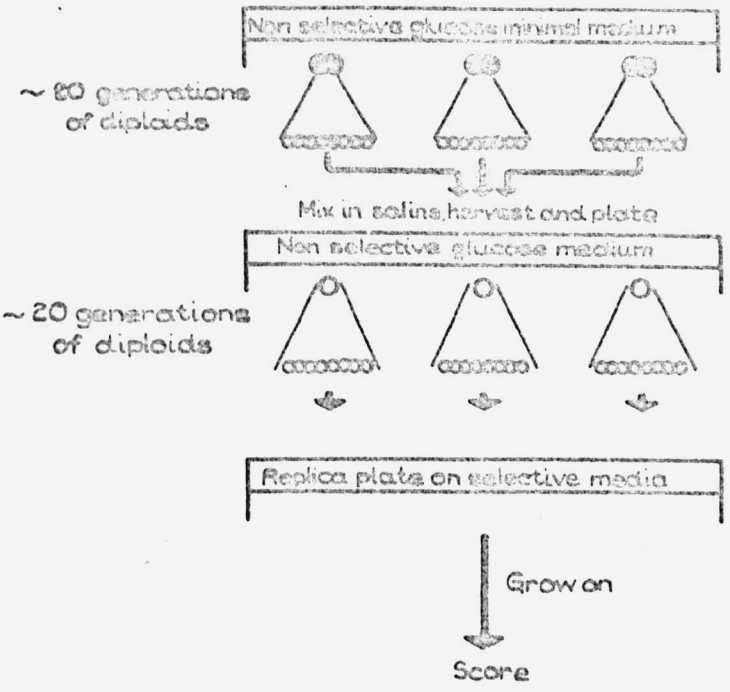
Analysis of Oligomycin Resistance
Transmission by Quantitative Replica Plating

After the diploids had been obtained as described above, the cells were suitably diluted so that on pipetting, 0.2 ml of the final diluted stock per plate (some 100-150 viable cells) was deposited. 0.2 ml of this stock was spread onto MMGlu plates and these plates incubated for 3 days at 30°C before replicating onto YEPGly plates with and without oligomycin. Plates were scored after 3 days incubation at 30°C. Exactly the same procedure was adopted to assay for erythromycin resistance transmission and also in the two- and three-point crosses performed both here and in the laboratory of Professor P. P. Slonimski. In the case of such three-point crosses between oligomycin, erythromycin and chloramphenicol resistances, replication was, of course, carried out using not only drug plates with just a single drug in it, but also the double media containing chloramphenicol and erythromycin or oligomycin respectively. These two procedures, quantitative aliquot plating and replica plating, are compared and illustrated in Figure 2.1.

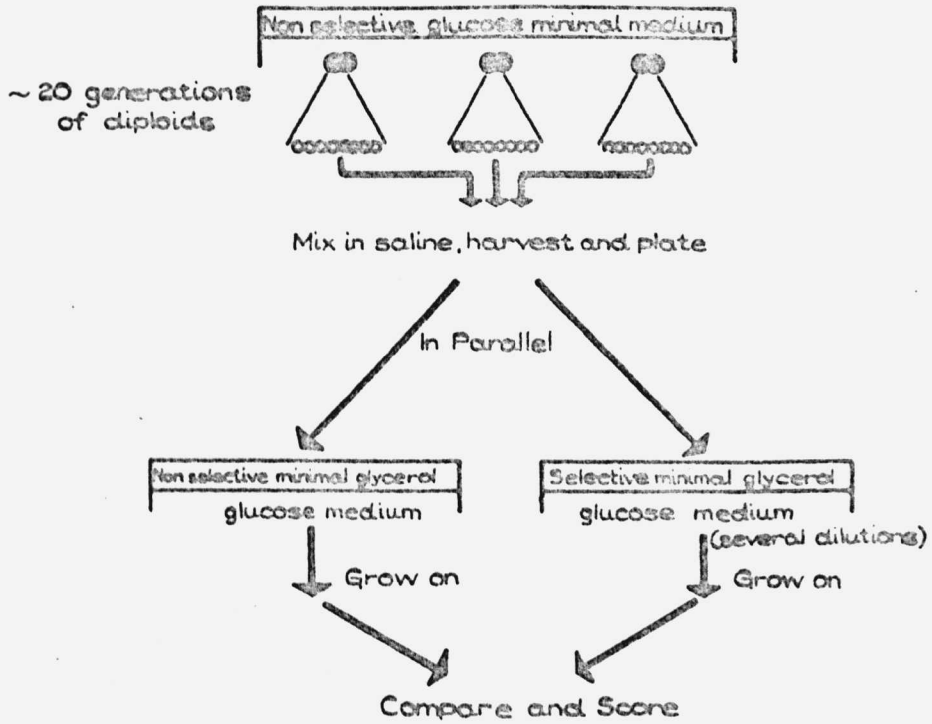
Procedure for Obtaining Synchronised Zygotes

The procedure used is that described by Coen et al. (Coen et al., 1970) and is a modification of that used by Jakob (1962). Strains D6 and D22B9 were purified and then grown on YEPG for 24 hours. The strains were then centrifuged, washed once with sterile H_2O and then resuspended in the same volume of H_2O as the culture medium they were grown in. About 10^6 - 5×10^6 cells of each of

QUANTITATIVE REPLICA PLATING



QUANTITATIVE ALIQUOT PLATING



the haploid strains were then inoculated into 10 ml of medium (1% yeast extract, 1% peptone, 0.05M Na^+ phosphate buffer, pH 6.25) and shaken for 90 - 120 minutes at 28°C. The culture was then centrifuged for 5 minutes at 2,000 G in a conical centrifuge tube and the cells left pelleted at the bottom of the tube at room temperature for 30 minutes. After this they were resuspended in the same medium as before (1% yeast extract, 1% peptone, 0.05M Na^+ phosphate buffer, pH 6.25) and again shaken vigorously for 90 - 120 minutes. After chilling in ice, the suspension was washed with cold saline by centrifugation. The pellet was suspended in 10 ml of sterile H_2O. It was this suspension which was either immediately plated out onto MMGlu to enable an analysis of zygotic clones to be made, or was inoculated into liquid MMGlu to enable the kinetic analysis of the oligomycin resistance loss on diploid formation from the class I mutant to be studied.

Procedure for Obtaining ρ^- Cells

Petites were obtained normally by culturing the strain from which petites were required in liquid YEPG in the presence of either 10.0 γ/ml ethidium bromide or 2.5 γ/ml acriflavine. The cultures were incubated at 30°C for 48 hours, centrifuged and washed with distilled H_2O before plating onto YEPG medium and incubating at 30°C for 4 days. Petites were identified by replica plating onto YEPGly, incubating at 30°C for 3 - 4 days and isolating the colonies which failed to grow on the latter medium. Alternatively, cells were plated directly onto PDM and the ρ^- colonies identified after 4 days at 30°C by their small size. Petite identification is aided in the case of strains derived from D22 in that they also lose the red colour associated with the ad_2 locus.

Genetic Analysis of ρ^- Cells for Suppressiveness

ρ^- cells were crossed to ρ^+ cells of the opposite mating type and the diploid cells so obtained plated onto PDM medium and incubated for 4 days at 30°C, before the percentage petites were scored. As this figure is a composite of the suppressiveness of the haploid ρ^- and the spontaneous petite mutational rate of the diploid, the latter was evaluated by crossing a ρ^+ cell from the same strain as the ρ^- was obtained, by the identical tester strain and analysing these diploids, as before, for their percentage petite frequency. The percentage suppressiveness was taken to be the former value minus the latter.

Genetic Analysis of ρ^- Cells for
Loss of Oligomycin Resistance

ρ^- strains were crossed by a $\rho^+ OL^S$ strain of opposite mating type and the resulting diploids tested for their oligomycin resistance either by dropping out directly onto YEPGly medium + oligomycin or by quantitative aliquot plating. The former method does not require purification of the diploids to free them of any viable haploids surviving the mating procedure as the ρ^+ haploids will not grow in the presence of oligomycin and the ρ^- haploids will not, as pointed out above, grow on glycerol anyway.

Tetrad Analysis

Diploids obtained by the procedures already described, were plated out to give single colonies. The resistance or sensitivity of these to oligomycin was evaluated by replica plating and individual colonies taken and grown in 10 ml pre-sporulation medium for 36 - 48 hours at 30°C. The tubes were occasionally shaken and after 36 - 48 hours, cells were centrifuged, washed twice with distilled water, and resuspended in 0.5 ml distilled water. A small amount of this suspension - 0.01 ml - 0.05 ml - was spread onto sporulation medium and incubated at 25°C for 3 - 5 days (Fowell, 1969). Asci for tetrad analysis were digested using a x 10 diluted preparation of the commercial snail gut enzyme preparation for some 20 - 40 minutes at 30°C. Dissection was carried out with a Singer micromanipulator. All other procedures were standard and are described by Fowell (Fowell, 1969) and Mortimer and Hawthorne (Mortimer and Hawthorne, 1969).

Random Spore Isolations

Random spore isolations have been carried out using essentially the method described by Fowell (Fowell, 1969), combining the digestion of asci using the snail enzyme (0.01 ml of the commercial enzyme preparation to 1 ml of a dense spore suspension, the whole incubated at room temperature overnight) followed by a homogenisation treatment using a tight-fitting glass homogeniser, with an enrichment step for the spores using a paraffin oil treatment (Emeis, 1958 ; Emeis and Gutz, 1958). In this step the homogenised spore suspension was shaken with paraffin oil and the paraffin layer allowed to separate from the water layer which was then discarded. The paraffin layer was then washed several times with an equal volume of water, each time shaking vigorously to mix the two phases, followed

by their separation and the retention of the paraffin layer. This washed suspension was then streaked out onto YEPG medium and incubated at 30°C for 3 days when the colonies were typed for segregation of both their drug resistance and nuclear markers.

Estimation of the Percentage Petite Frequency

Three procedures were used. Firstly, cells were plated directly onto PDM medium and incubated for 4 days at 30°C, followed by scoring for the large and small colonies the latter corresponding to the petites. Secondly, cells were plated directly onto YEPG plates grown at 30°C for 2 3 days, then replicated onto YEPGly medium and the colonies which grew on the former, but not the latter, scored as petites. Thirdly, the tetrazolium overlay technique has been used (Ogur et al., 1957 ; Nagai, 1959). In the case of D22 and derived strains, subsidiary estimations of petite frequency could be made by noting the loss of the red colour characteristic of this ad_2 mutant, this loss being associated with petite formation. In none of these procedures has any distinction been made between different types of mutants unable to grow on non-fermentable substrate and not showing electron transport ability.

Cell Growth Rate Estimations

(a) Optical Density

The optical density of cultures was measured using an EEL colorimeter fitted with a 607 filter (red). Cultures were read against a culture medium blank. In order to simplify sterility problems, specially made conical flasks with sidearms that fitted exactly the optical cavity of the EEL were routinely used as growing vessels for the yeast strains. A calibration curve of O.D. vs. dry weight was obtained.

(b) Dry Weight

Dry weight determinations were carried out on cells which had been washed with distilled water twice. The cells were dried at 105°C for 3 days in pre-weighed vessels.

Whole Cell Respiration Studies

Yeast cells grown as described in the text were centrifuged, washed once with 0.1 M phosphate buffer, pH 7.0, and resuspended at high concentration in this same buffer with added glucose or ethanol (0.2% final concentration). Respiration was assayed using a Rank electrode, fitted to a Gilson recording oxygraph. The total volume of additions to the electrode cells was 3.0 ml. Assays were carried out at 30°C.

Growth Yield Studies

Cells were normally grown in YEP ± various amounts of ethanol ranging from 0.05 - 0.5% final concentration, using a 0.2% initial inoculum of stationary phase YEPG grown cells. Unless otherwise stated, 100 ml of medium was used in each 500 ml conical sidearm flask and the cultures grown at 30°C on a Gallenkamp rotary shaker (speed 250 rev/min). Cells were removed from all the flasks when the O.D. of the flask containing the highest concentration of ethanol was constant, split into two 50 ml aliquots and dry weight determinations made on these parallel samples as detailed overleaf.

Growth Conditions for Cells Taken for Electron Microscopy

The strains to be used for electron microscopy were grown normally at 30°C in bubble tubes containing 10 ml of YEP 1% EtOH to which had been added one drop of antifoam. A solid inoculum was normally used and the cells grown for 12 - 20 hours, by which time they were in early to mid-log phase. $^{\Delta}$OD's were taken to check this fact prior to samples being taken for electron microscopy. Oligomycin, when added to the cultures, was added at the start of the growth period and allowance made for the ethanol added at the same time.

Preparation of Spheroplasts for Electron Microscopy

The procedure used was essentially that described by Kovác et al. (Kovác et al., 1968a) but the cells from each growth tube were resuspended in 2.5 ml of the digestion buffer and the high concentration of 0.2 ml of the crude Helix pomatia enzyme added and incubation continued at 30°C for 30 - 45 minutes. The spheroplasted yeast cells were then spun down and either used directly for electron microscopic examination or washed once with 1 M Sorbitol 0.01 M citrate phosphate buffer, pH 5.8 and 10 mM EdTa prior to this.

CHAPTER 3

GENERAL GENETIC CHARACTERISATION OF *S.cerevisiae* STRAINS D6 AND D22

Introduction

In this chapter the results of the characterisation of the response of the wild type strains D6 and D22 to $CoSO_4$ and to both nitrous acid and u.v. mutagenesis are presented. In addition, results of preliminary investigations into the utility of various enrichment techniques for the isolation of mitochondrial and, more specifically, oxidative phosphorylation mutants are detailed.

Enrichment procedures used in the isolation of yeast mutants include : nystatin (Moat et al., 1959; Snow, 1966; Stromnaes and Mortimer, 1968), cycloheximide (Moat et al., 1959; Pittman et al., 1963), endomycin (Moat et al., 1959; Stromnaes and Mortimer, 1968), 'inositol less' death (Megnet, 1964) and deoxyglucose (Megnet, 1965(a); Megnet, 1965(b)) procedures. These techniques have generally been used in connection with the isolation of either amino acid or vitamin auxotrophic strains. Their use in enriching for mutants of respiration and related pathways does not seem to have occurred.

The 'inositol less' death technique used in *Schizosaccharomyces pombe* is a variant on the so called 'lethal unbalanced' growth effect which has been utilised extensively in bacterial mutant enrichment procedures. The most widely used variation on this technique involves the use of penicillin, which, by preventing cell wall synthesis without inhibiting cell growth, leads to the cells literally 'bursting at the seams' (McQuillen, 1958). By adjustment of the experimental conditions, this property of penicillin has been used to select for particular classes of auxotrophic mutants (Davis, 1948; Lederberg and Zinder, 1948). Bauman and Davis (Bauman and Davis, 1957) observed that bacteria requiring either diaminopimelic acid or thymine for growth die rapidly if grown in media without these substrates. Bacteria which carry additional auxotrophic markers, on the other hand, show a greatly reduced death rate. This effect has formed the basis of another enrichment technique.

Unbalanced growth enrichment techniques have also been employed with

fungi. Apart from the use of the 'inositol less' death procedure in Schizosaccharomyces (Megnet, 1964), Fries has used similar effects of pyridoxine and thiamine requirements in Ophiostoma to enrich for particular mutant classes (Fries, 1948). Similar procedures have also been used by Holliday in Ustilago maydis and Pontecorvo et al. in Neurospora crassa (Holliday, 1962; Pontecorvo et al., 1953). The latter workers, in an extensive investigation, have shown that mutants auxotrophic for biotin (bi_1) die much faster than mutants auxotrophic for adenine (ad_1) when each strain is grown with its auxotrophic requirement unfulfilled. When an auxotrophic strain in which a second balancing mutation has been introduced was grown with both the auxotrophic requirements unfulfilled, its death rate approximated to that of a strain carrying the auxotrophic mutation causing the lowest death rate (Pontecorvo et al., 1953). Pontecorvo et al. (Pontecorvo et al., 1953) have suggested that starvation enrichment is more likely to be successful where the initial auxotrophic mutation does not have an immediate general growth suppressing effect (e.g. adenine) but a latent growth distorting effect (e.g. biotin). However, Fries (Fries, 1948) has successfully used thiamine auxotrophic strains in Ophiostoma. Despite the finding that in Neurospora adenine auxotrophic markers produce only small lethal growth effects, the parental strain D22 has been examined for such unbalanced growth effects.

The use of polyene antibiotics such as nystatin, endomycin and amphotericin B (Kinsky, 1967) in enriching for auxotrophic mutants in yeast, Penicillium chrysogenum and Neurospora crassa is well established (Ditchburn and MacDonald, 1971; Hopwood, 1970; Snow, 1969; MacDonald, 1968; Wills, 1968). These antibiotics apparently affect the permeability of the cell membranes causing the wholesale loss of soluble cellular components (Kinsky, 1967). This effect is reflected in the great variety of metabolic processes which are inhibited by these polyene antibiotics in the intact fungal cell (Kinsky, 1967). The insensitivity of bacteria to these antibiotics has been related to the non-absorption of the antibiotic and specifically to the lack of uptake due to the absence of sterols from their cell membranes (Kinsky, 1967). Their use as selective enriching agents is related to their greater effectiveness and presumably uptake into growing than into non-growing cells. Thus, if conditions can be arranged so that the desired class of mutants ceases growth whilst that of the wild type

continues, then the drug will generally selectively kill the growing wild type cells whilst enriching the remaining viable cells for the desired auxotroph. The action of nystatin is known to be both pH (Lampen et al., 1959) and temperature dependent, and it has also been reported that it is influenced by the presence of metabolisable substrate (Ghosh and Ghosh, 1963a,b; Lampen et al., 1959). The effectiveness of nystatin as an enrichment agent for mitochondrial mutants has been examined using reconstruction experiments involving petite and grande cells.

Results and Discussion

U.V. mutagenesis of S.cerevisiae D6 and D22

The procedure followed was that documented in chapter 2. Following irradiation for various times, cells were plated out onto YEPG medium and incubated for four days at 30°C when they were scored for total colony number and also for the percentage petite frequency, using the tetrazolium overlay technique. The results are shown in figures 3.1 and 3.2. It can be seen that there appears to be a very rapid increase in petite frequency with irradiation for D6 and a comparatively slow rise following a lag period for D22. In view of the number of cells counted and the much smaller spontaneous petite frequency of D22, making petite estimates less reliable, the significance of this is uncertain. Figure 3.2. shows that after 60 - 80 seconds maximal mutational efficiency as measured by reversion at the ad_2 locus has been achieved. In view of the findings, 60 - 80 second irradiation periods have been used routinely.

Nitrous acid mutagenesis - characterisation of strain response

Figure 3.3. shows the survival curve of S.cerevisiae D6 when treated with nitrous acid using the procedure outlined in chapter 2. Similar curves had previously been obtained in experiments with S.carlsbergensis 74S. Trial experiments using various starvation periods prior to mutagenesis were shown to make no appreciable difference to the efficacy of mutagenesis in these strains. Overnight dark storage at 0°C prior to spreading of strains which had been resuspended in phosphate buffer pH 7.3. after mutagenesis resulted in a decrease in cell viability in comparison to cells spread immediately after mutagenesis (see Figure 3.3).

Characterisation of the effect of $CoSO_4$ on S.cerevisiae D6 and D22

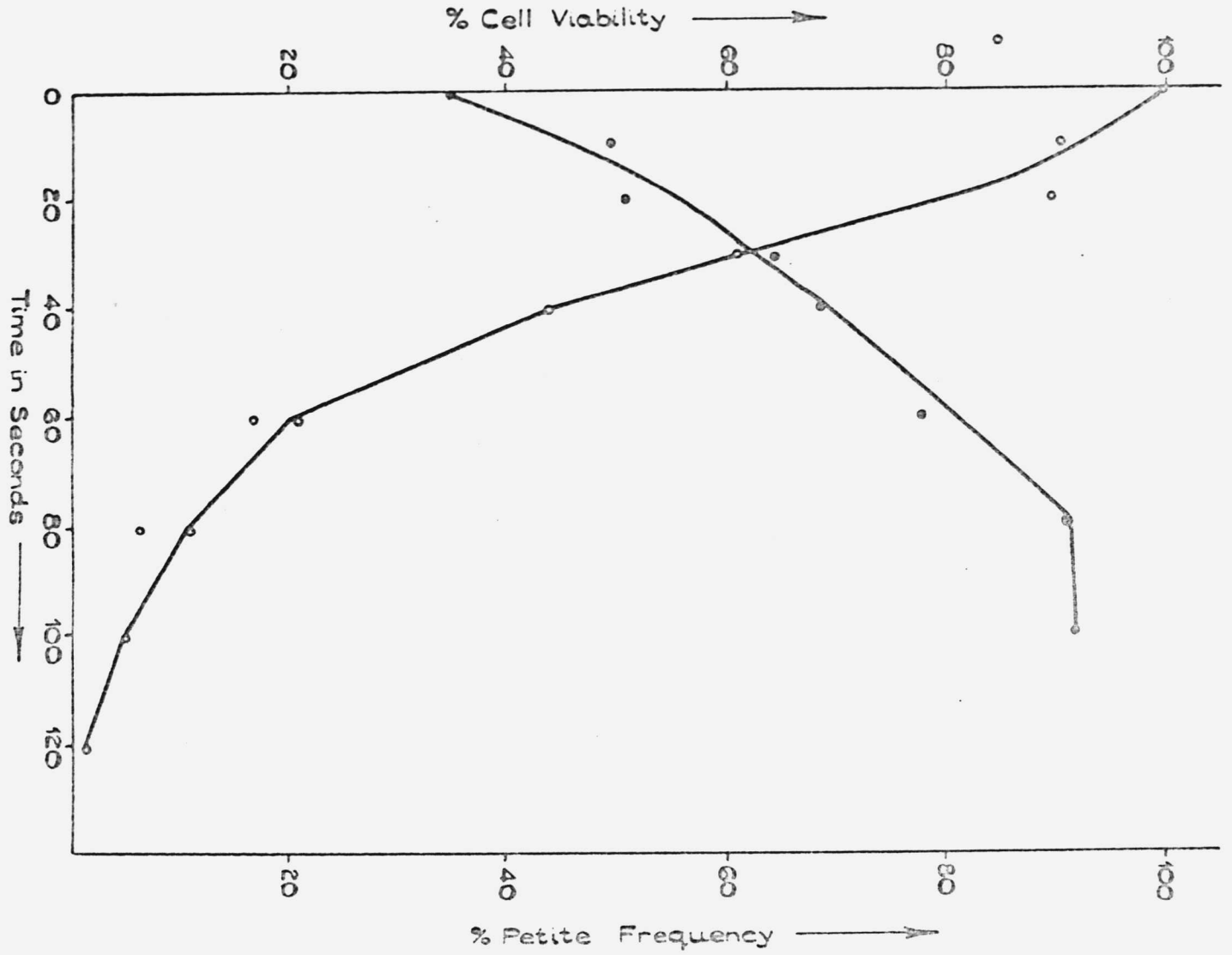
Heavy metals, including cobalt sulphate, are highly effective at ~~inducing~~ selecting

LEGEND FOR FIGURE 3.1.

U.V. Mutagenesis of D6

S.cerevisiae D6 was grown aerobically in liquid YEPG medium at 30°C. These cells were subjected to u.v. mutagenesis using the procedure outlined in chapter 2. Aliquots taken after various periods of mutagenesis were spread onto YEPG plates and incubated at 30°C. Cell viability and percentage petite frequencies were scored after 4 days incubation at 30°C, the latter using the tetrazolium overlay technique.

- % petite frequency of total viable cells
- ○ % cell viability of total cells plated.

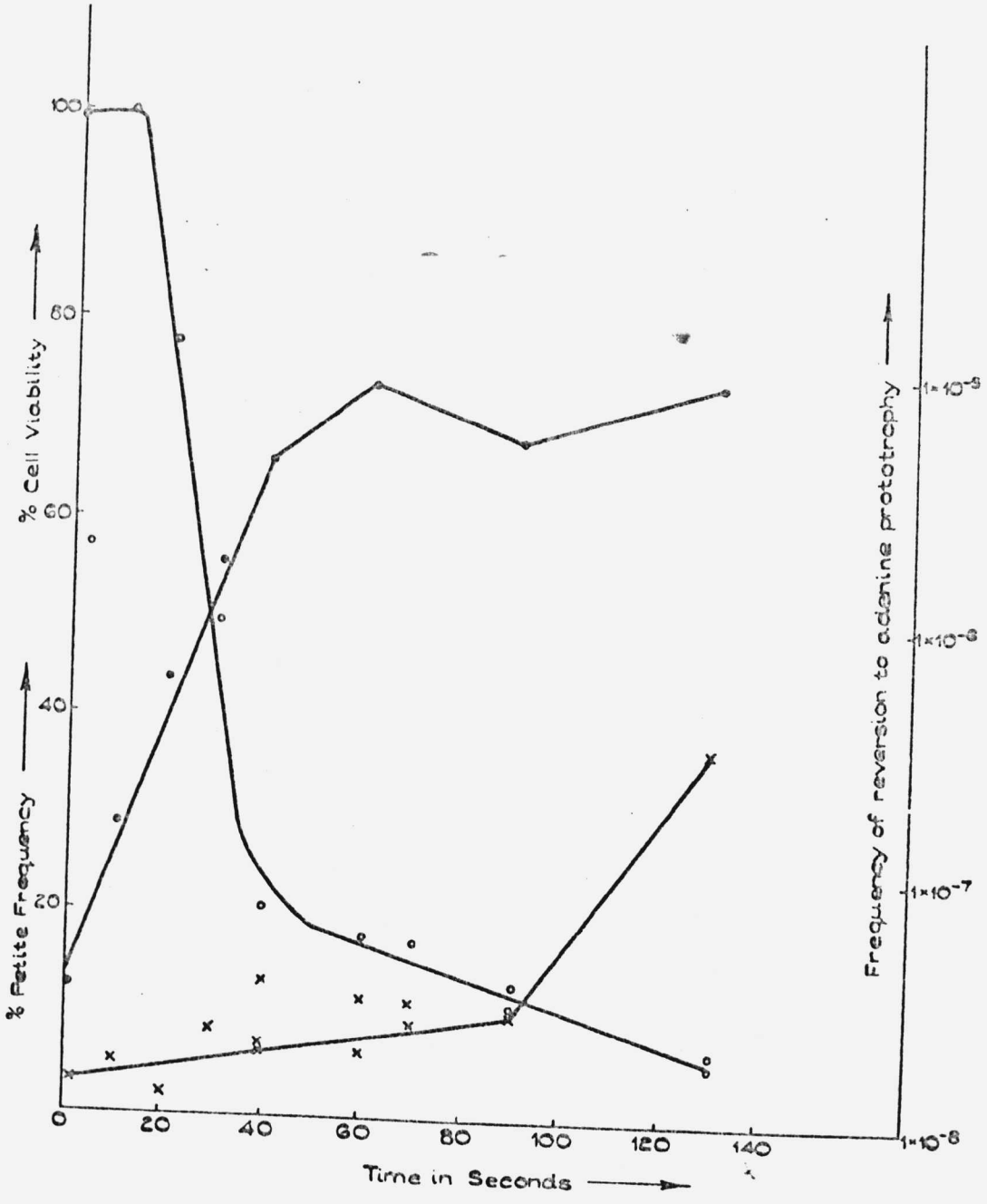

% Cell Viability
20
40
60
80
100
0
20
40
60
80
100
120
Time in Seconds
20
40
60
80
100
% Petite Frequency

LEGEND FOR FIGURE 3.2.

U.V. Mutagenesis of D22

S.cerevisiae D22 was grown aerobically in liquid YEPG medium at 30°C. These cells were subjected to u.v. mutagenesis using the procedure outlined in chapter 2. Aliquots taken after various periods of mutagenesis were spread onto MMGlu and YEPG plates and incubated at 30°C. Cell viability and percentage petite frequency were estimated by scoring the YEPG plates after 3 days incubation at 30°C, the latter using the tetrazolium overlay technique. ad_2 revertants were estimated by scoring the number of colonies growing on the MMGlu plates after 4 days.

- × % petite frequency of total viable cells plated
- o % cell viability of total cells plated
- • % frequency of ad_2 revertants amongst viable cells plated.

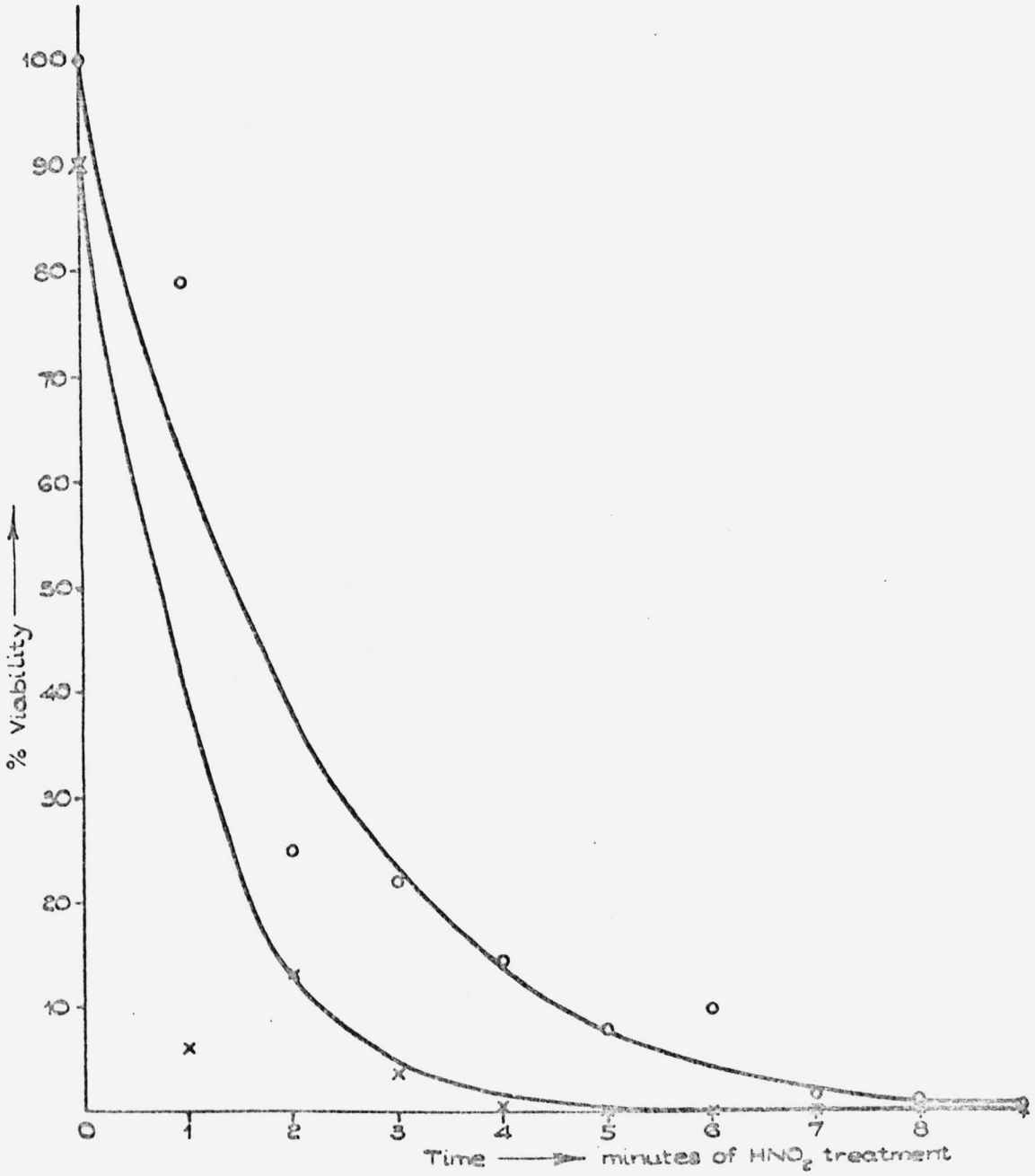

% Cell Viability
% Petite Frequency
Frequency of reversion to adenine prototrophy
Time in Seconds
100
80
60
40
20
0
20
40
60
80
100
120
140
1×10⁻⁵
1×10⁻⁶
1×10⁻⁷
1×10⁻⁸

LEGEND FOR FIGURE 3.3.

HNO_2 Mutagenesis of D6.

S.cerevisiae D6 was grown aerobically in liquid YEPG at 30°C. These cells were subjected to nitrous acid mutagenesis using the procedure outlined in chapter 2. Aliquots taken after various periods of mutagenesis were spread on YEPG and incubated for 3 - 4 days prior to scoring for cell viability.

- ○ % cell viability (spread immediately after mutagenesis)
- × % cell viability (held 16 hours in dark at 0°C prior to spreading)


100
90
80
70
60
50
40
30
20
10
% Viability
0
1
2
3
4
5
6
7
8
Time
minutes of HNO_2 treatment

petites (Horn and Wilkie, 1966; Lindegren et al., 1958). Strains D6 and D22 have been checked for both their sensitivity to $CoSO_4$ and for its effect on petite selection. The $CoSO_4$ was added to the medium after sterilising and the overnight YEPG grown cell of the two strains plated out after suitable dilution. Plates were incubated at 30°C for four days and the petite frequency scored by the tetrazolium overlay method. The results are shown in Figures 3.4 and 3.5. D6 appears very resistant to the effects of $CoSO_4$ - both as exhibited by its failure to ~~induce~~ select petites and also, at least within the concentration ranged used, to affect the cell viability. D22, on the other hand, is sensitive to concentrations of $CoSO_4$ higher than 1.75 - 2 mM and the sensitivity of petite ~~induction~~ frequency shows a complete coincidence with the effect exhibited on cell viability, the former rising whilst the latter falls.

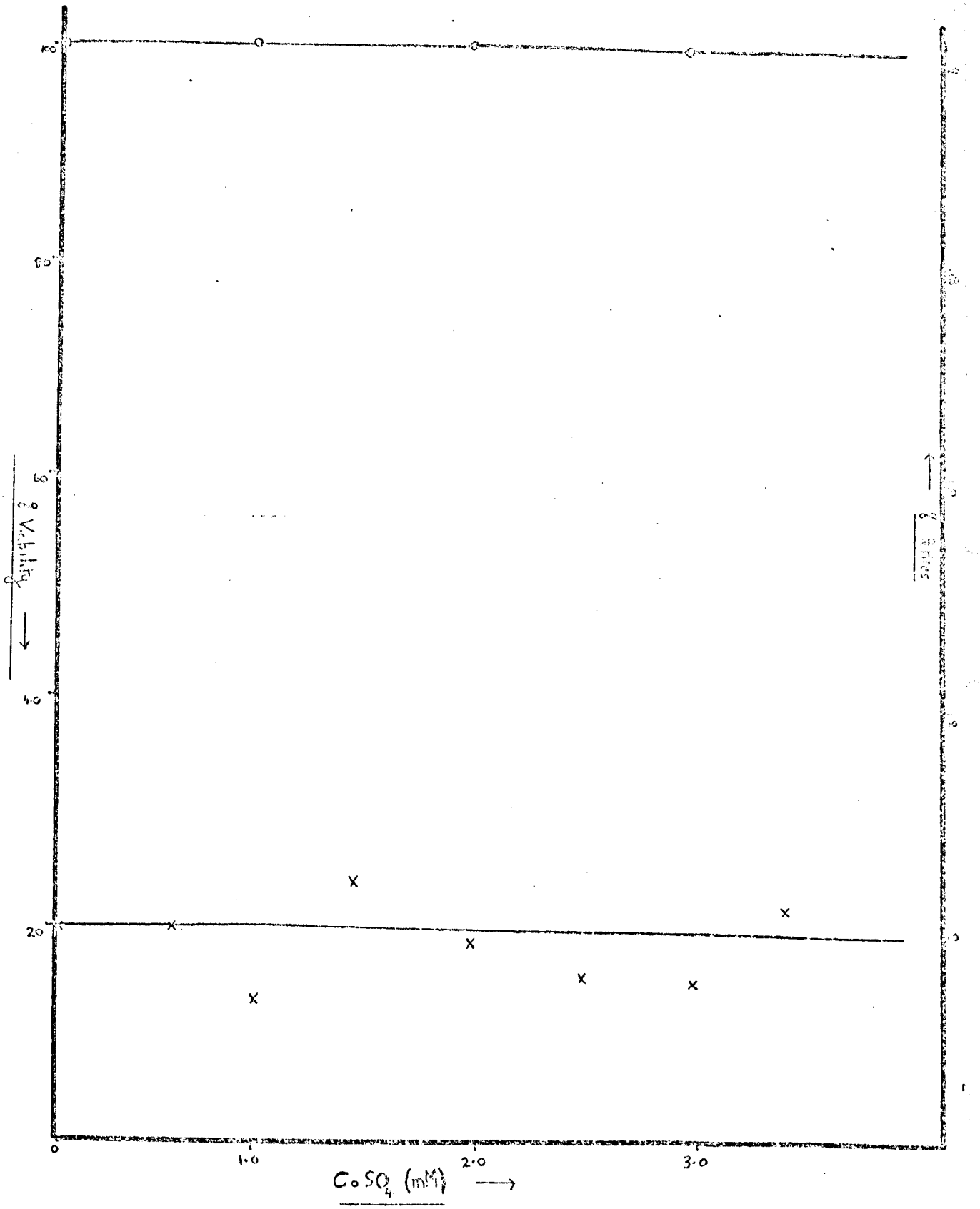
Characterisation of possible enrichment procedures

Reconstruction experiments have been carried out to explore the efficacy of the starvation growth enrichment technique used by Megnet (Megnet, 1964) as a means of enriching for mutants of S.cerevisiae with defective mitochondrial metabolism. D22 (adenine requiring wild type) and a spontaneously derived petite, D22 ρ -1, were plated out at two cell densities onto either glycerol or glucose medium in the absence of adenine. At various times their level of survival was checked by adding adenine + glucose to the plates. It was assumed that the effects the ρ-mutation produced on cellular metabolism would be similar to those produced by an oxidative phosphorylation mutant and that this was therefore a valid 'reconstruction' study of the possible utility of the selection procedure. The results are shown in Figure 3.6. The survival curves of both D22 and D22 ρ -1 on MMGlu are similar and neither strain appears to lose viability in the absence of adenine over a period of nine days. It therefore appears that the absence of adenine from the cell does not lead to 'unbalanced cell' growth and premature death due to adenine starvation. Lack of respiratory capacity causes the ρ-strains to show a sharp fall in viability with time of starvation when they are plated on MMGly medium. This result suggests that even if a suitable locus for causing 'unbalanced growth' can be found in S.cerevisiae, it may not be practical to use it for isolating mitochondrial mutants as the enrichment procedure will require starvation on a medium containing only non-fermentable carbon sources, and this apparently leads to the death of cells of the ρ-type, and by analogy, to that of other mutants deficient in functional

LEGEND TO FIGURE 3.4. $CoSO_4$ Treatment of

○ % viable colonies of cells plated

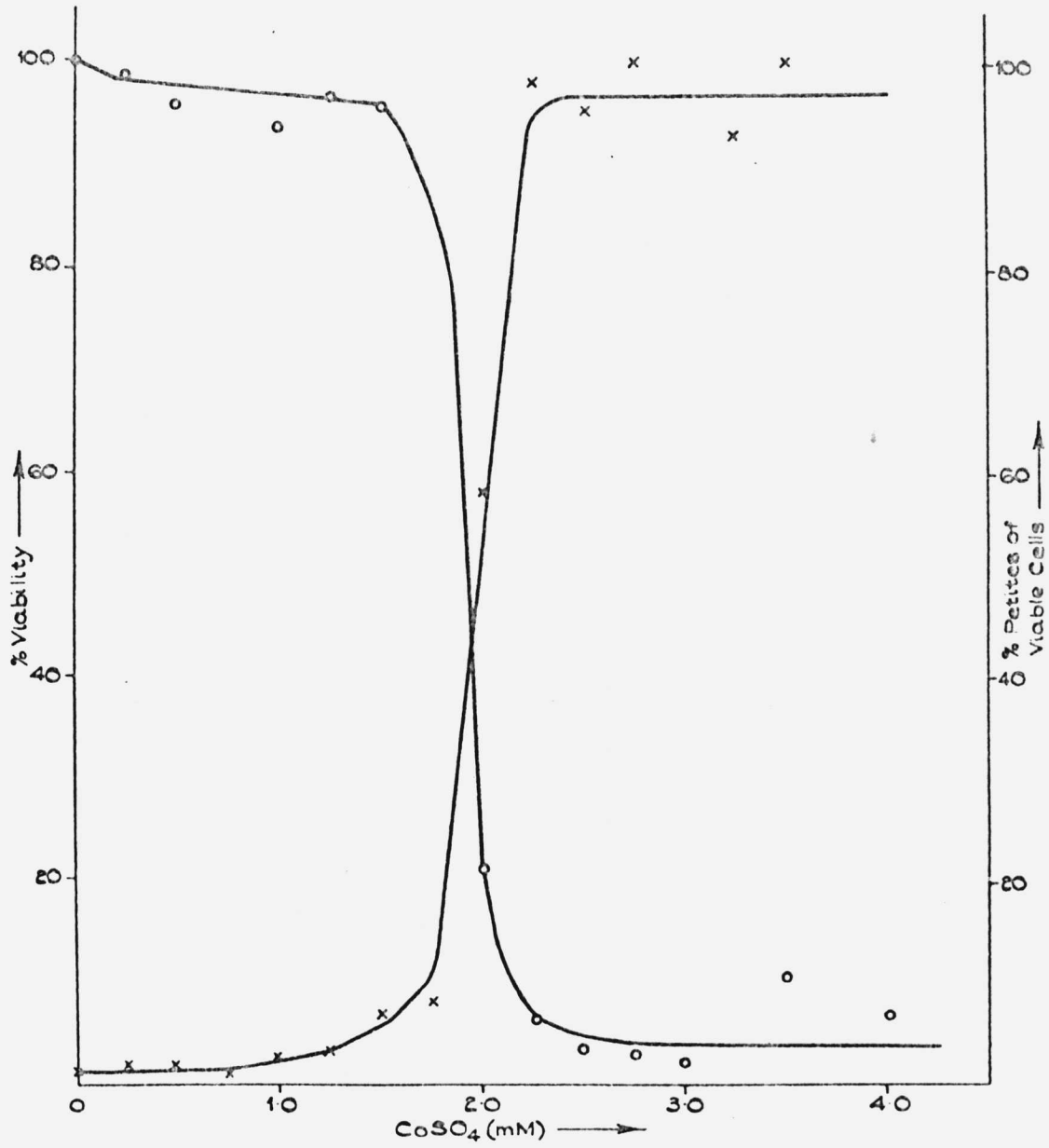
× % petite colonies of total viable colonies


100
80
60
40
20
0
Viability %
1.0
2.0
3.0
CoSO4 (mM)

LEGEND TO FIGURE 3.5. $CoSO_4$ Treatment of D22

○ % colony viability of cells plated

× % petite colonies of total viable colonies

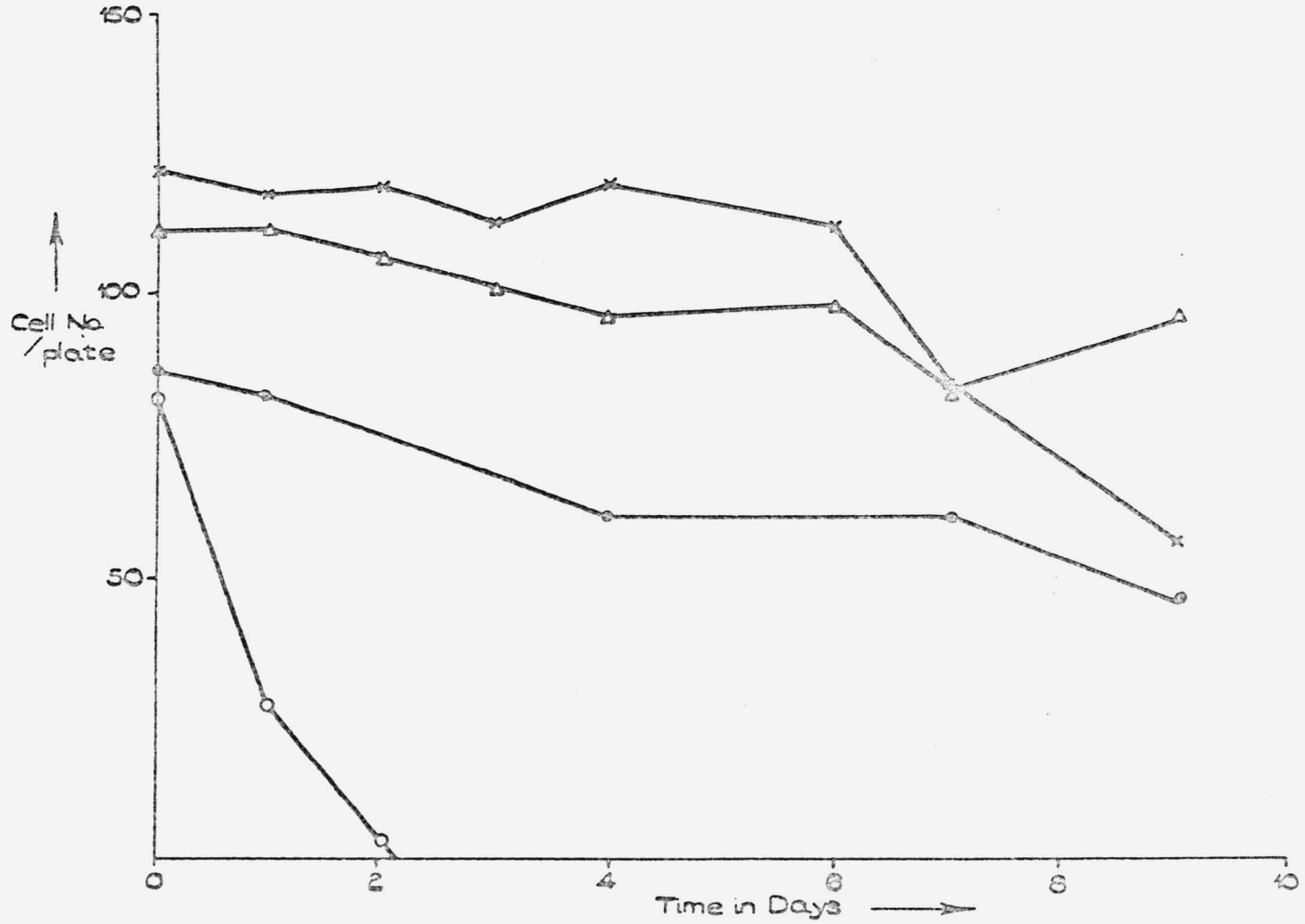

100
80
60
40
20
% Viability
% Petites of Viable Cells
0
1·0
2·0
3·0
4·0
CoSO4 (mM)

LEGEND FOR FIGURE 3.6.

D22 ρ^-1 and D22 were both grown in YEPG liquid medium at 30°C until they reached stationary phase when they were plated out onto MMGly and MMGlu plates at two different cell densities - 100 and 1000 cells per plate. (The MMGly and MMGlu agar plates were thick to facilitate lifting). The plates were incubated at 30°C and after 0, 1, 4, 7 and 9 days plates of both strains, on each media and at both cell concentrations were taken, the agar lifted from the petri dish with a sterile spatula and 1 ml of adenine + glucose solution (300 mg/ml glucose ; 0.2 mg/ml adenine) injected through the agar with a sterile syringe, after which the agar was replaced. The plates were reincubated at 30°C and scored after a further 4 days for the total number of viable colonies.

Total Viable Colonies

- ○ D22 ρ^-1 grown on MMGly prior to adenine/glucose addition
- ● D22 ρ^-1 grown on MMGlu prior to adenine/glucose addition
- Δ D22 grown on MMGly prior to adenine/glucose addition
- × D22 grown on MMGlu prior to adenine/glucose addition


150
100
50
Cell No /plate
0
2
4
6
8
10
Time in Days

mitochondrial activity. Under these circumstances the 'balancing' effect of a strain carrying these two mutations rather than the single one leading to unbalanced growth will not be apparent and enrichment cannot occur. A similar finding that starvation can adversely affect the survival of auxotrophic strains in comparison to prototrophic ones has been recently reported in studies on Neurospora crassa (Ditchburn and MacDonald, 1971). After the experiments were completed, Snow (Snow, 1969) reported that this technique did not work efficiently on S.cerevisiae even when an inositol requiring strain was used and attempts made to enrich for amino acid auxotrophs.

An alternative technique to that described above involves the use of nystatin as the enrichment agent. Support for the idea that the nystatin selection technique might be applicable to mitochondrial mutant enrichment was given by the findings of Snow (Snow, 1966) that enrichment for other auxotrophic mutants with nystatin was simultaneously accompanied by enrichment for respiratory deficient strains. In these experiments of Snow (Snow, 1966) strains were grown with glucose as the carbon source and the enrichment for petites was presumably related to their slower growth rate. The selective enriching action of nystatin has been related to its greater uptake into growing cells (Lampen et al., 1959). The enrichment that Snow reported for petites was, however, not nearly as great as that found for the amino acid and vitamin auxotrophic mutants.

In view of these results 'reconstruction' experiments were undertaken with D22 and D22 ρ^-1 to see whether experimental conditions could be altered to provide a still more efficient enrichment for mitochondrial mutants. By analogy with the earlier work of Snow (Snow, 1966), it was decided to try to create conditions where the ρ^- cells would cease growth completely whilst the wild type ρ^+ would continue growth. The simplest system seemed to be to use the known biphasic growth of S.cerevisiae on glucose and to add nystatin to the culture when the cells were growing solely by oxidising the ethanol produced fermentatively from the glucose. Under these conditions the petite should not grow but the wild type grande cells will grow. When these experiments were carried out and nystatin added at a concentration of 10 γ/ml to yeast growing in unbuffered YEPG - initial pH 5.5 - 6.0 - there was a gradual drop in cell viability when this was assayed by plating out cells onto YEPG after various

times of treatment. By 40 minutes, however, cell viability had only dropped to 20% of the initial viable cell count and furthermore, no evidence of petite enrichment was apparent. In view of the evidence that the killing effects of nystatin are increased by lowering the pH, the experiments were repeated using YEPG medium but buffered at pH 4.0. In this case the killing effect was more rapid but again, little evidence of petite enrichment was obtained. In view of these results and the possibility that nystatin was complexing with the organic constituents of the YEPG media - its action is known to be antagonised by the presence of sterols and other lipids - identical experiments were performed using cells growing oxidatively in liquid MMGlu medium supplemented with adenine and buffered at pH 4.9 and 5.5. In both cases cell viability dropped quickly and within 30 minutes, less than 1.0 - 0.1% of the original cells were viable, but at most, a three- to five-fold enrichment for petites was achieved. The failure to observe any marked enrichment of petites in this experimental situation is puzzling in view of Snow's results (Snow, 1966). That the nystatin was active is shown by the fact that it was effective in killing the cells. A number of alternatives can be advanced to explain our results. Firstly, under the conditions used, the petites may be more sensitive to nystatin attack than the grandes despite their lowered or zero growth at the time of its addition. Ditchburn and coworker (Ditchburn and MacDonald, 1971) have suggested that certain amino acid auxotrophic strains have altered resistance to nystatin. It is, however, difficult to reconcile such an explanation with Snow's results. Secondly, as all the cells were growing on ethanol rather than glucose as in Snow's experiments, the growth rate of all the cells will be slower. If the petites are still showing some residual growth, the differential growth rate between grandes and petites may actually be less in our experimental situation than in Snow's. In this respect, the presence of high cell densities in the treated cultures, i.e. 1 - 2.5 x 10^8 cells/ml may be important as the cell lysis and release of cell metabolites caused by nystatin may lead to some cross feeding of fermentable substrates from ρ^+ to ρ^- cells. This might allow the latter to grow slightly, thus reducing the enrichment effect. Thirdly, nystatin may act in a different and possibly less selective manner on cells growing on ethanol as compared to cells growing on glucose. This could be

due to changes in either the metabolism or the chemical composition of the cells. It is known that the lipid composition of the yeast cell is markedly dependent on growth condition (Paltauf and Johnston, 1970; Kates and Baxter, 1962; McMurrough and Rose, 1971) and that the action of nystatin involves a sterol component in the membrane (Kinsky, 1967). As Lampen et al. (Lampen et al., 1959) and Ghosh and Ghosh (Ghosh and Ghosh, 1963, a,b) have reported that the initial uptake of the antibiotic is probably an energy process which is stimulated by metabolisable substrate, there is, in addition, the possibility that the nature of this substrate itself directly influences the efficiency of the nystatin attack.

CHAPTER 4

EFFECT OF OLIGOMYCIN ON THE GROWTH OF S.cerevisiae AND THE ISOLATION AND CHARACTERISATION OF THE OLIGOMYCIN RESISTANT MUTANTS

Introduction

Attempts to obtain oxidative phosphorylation mutants in S.cerevisiae by isolating strains with functional electron transport, as demonstrated by their ability to reduce tetrazolium dyes, and yet unable to grow on non-fermentable media, have been made by several groups of investigators. Kovác, Lachowitz and Slonimski succeeded in isolating the op_1 mutant (Kovác et al., 1964) whilst Beck et al. (Beck et al., 1968) have isolated the p_9 mutant - these two mutants subsequently appearing to be probably identical. Parker and Mattoon (Parker and Mattoon, 1969) have also isolated some presumptive oxidative phosphorylation mutants using this technique. In many cases, however, the phenotypes of the mutants are ill defined, the levels of the cytochromes being altered even though respiratory activity is retained (Parker and Mattoon, 1969). The biochemical analysis of the most widely investigated mutant has also proved disappointing (Beck et al., 1968; Kovác et al., 1964; Somlo, 1970; Somlo, 1971; Kovác et al., 1968a) at least in-so far as it has failed to materially increase our knowledge of the enzymic processes involved in oxidative phosphorylation.

Investigations centering on a single mutant have, of course, built-in limitations and the value of systematically studying a series of mutants whose genetic relationship to each other is known has been amply demonstrated by the studies of bacterial ribosomes (Dekio and Takata, 1969; Dekio et al., 1970; Tanaka et al., 1968) and in yeast by Sherman and Margoliash and coworkers in an extensive study of cytochrome c mutants (Sherman et al., 1970; Mattoon and Sherman, 1966; Sherman and Stewart, 1971).

Studies of oxidative phosphorylation mutants when obtained using total isolation methods may be hampered by the number of mutants possessing the desired phenotype due to a phenotypic change which is not primarily related to

the energy conservation and ATP synthesis process (Kovác and Hrusovská, 1968).

In view of all these facts, two other approaches to the problem appear worthwhile. Firstly, the isolation of mutants resistant to drugs whose known locus of action is at the mitochondrial ATP synthetase complex, and their subsequent rescreening to exclude mutants whose phenotype is due to a change in cell permeability or mitochondrial permeability or to a cellular detoxifying system. This approach may be coupled with a secondary screening for temperature sensitivity. Secondly, the isolation of temperature sensitive mutants which lose their ability to grow on non-fermentable substrate only at the non-permissive temperature. This approach has been used by Weislogel and Butow (Weislogel and Butow, 1970) amongst others, but will obviously result in the isolation of mutants temperature conditional for other activities such as mitochondrial protein synthesis, mitochondrial RNA synthesis, etc., as well as for respiration and oxidative phosphorylation.

For these reasons, as a primary approach to the problem of isolating mutants possessing lesions in oxidative phosphorylation metabolism, oligomycin and chloro CCP resistant mutants have been isolated. In the following five chapters of this thesis, the results obtained with the oligomycin resistant mutants are presented. At the start of this investigation only one report of the isolation of oligomycin resistant mutants had appeared in the literature (Parker et al., 1968) though subsequently two other papers relating to such mutants have appeared (Stuart, 1970; Wakubayashi and Gunge, 1970). The need for a seriously correlated morphological biochemical and genetic approach to the problem has, however, remained.

Results

Effect of oligomycin on the growth of S.cerevisiae wild type strains D22 and D6

The specificity of action of oligomycin on intact yeast cells was checked prior to its use in isolating resistant mutants. Figure 4.1. shows that when 5.0 γ/ml of oligomycin was added to the wild type strain D22 growing logarithmically with glycerol as the energy source and therefore wholly dependent on mitochondrial respiration for energy generation, growth was completely inhibited. In contrast, no growth inhibition was observed when an

LEGEND TO FIGURE 4.1.

Inhibitory Effects of Oligomycin

A 1% inoculum of D22 (YEPGly grown) was inoculated into 100 ml of YEPGly media in 500 ml sidearm flasks and the flasks grown at 30°C. 5.0 γ/ml oligomycin in an ethanolic solution or an equivalent amount of EtOH were added to the flasks as indicated. The growth rate was followed using an EEL colorimeter fitted with a 607 filter.

Key

• + 5.0 γ/ml oligomycin

× + EtOH

LEGEND TO FIGURE 4.2.

Inhibitory Effects of Oligomycin

1 ml of a D22 starter culture (YEPG stationary phase) was inoculated into sidearm flasks containing 100 ml of YEPG and the cultures incubated at 30°C. At the point indicated either 5.0 γ/ml of oligomycin in ethanolic solution or an equivalent amount of ethanol (0.2%) was added to the flasks. The growth rate was followed using an EEL colorimeter fitted with a 607 filter.

Key

• + 5.0 γ/ml oligomycin

× + EtOH

Figure 4.2

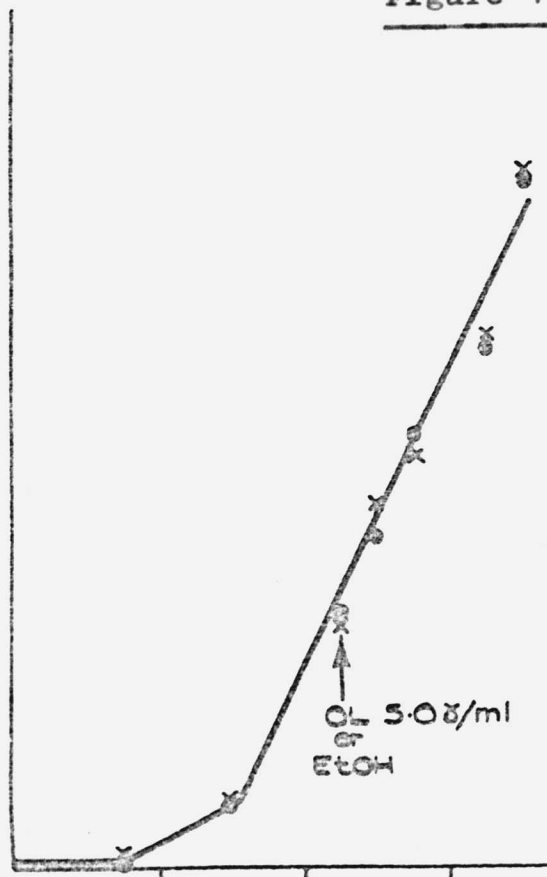


Figure 4.1

equal concentration of oligomycin was added to similar mid-log phase cultures which were growing fermentatively with glucose as the carbon source (Figure 4.2). The inhibitory effect of oligomycin is therefore totally restricted to an inhibition of growth dependent on mitochondrial metabolism for energy conservation. This suggests that oligomycin is probably acting in the same mode in vivo as it does in vitro in inhibiting mitochondrial ATP generation (Huijing and Slater, 1969; Slater and Ter Welles, 1969). The specificity of oligomycin was confirmed by the experiment illustrated in figure 4.3. when oligomycin was added simultaneously with the yeast inoculum to cultures growing in YEPG medium. Whilst the secondary non-fermentative phase of growth was totally inhibited by oligomycin, the primary fermentative phase was unaffected. There is some indication that growth during the fermentative phase is slightly slower in the oligomycin treated culture than in the control culture. The effect, however, is very small and appears to be due to some residual mitochondrial function remaining non-glucose repressed during parts of the fermentative growth phase and contributing to a minor extent to the cellular energy pool.

Figure 4.4. shows the cell yield of S.cerevisiae grown on YEPG in the presence and absence of various concentrations of oligomycin. Concentrations of between 0.63 and 1.25 γ/ml oligomycin are necessary to inhibit the non-fermentative phase of yeast metabolism. No intermediate levels of growth inhibition were obtained and oligomycin appears to produce an 'all or none' inhibition of growth. The levels of oligomycin necessary to inhibit oxidative metabolism in liquid media correlate completely with the concentrations necessary to inhibit growth on YEPGly solid medium when 0.5 and 0.75 γ/ml oligomycin are needed to inhibit strains D22 and D6. Similarly, lack of inhibition of fermentative growth in liquid YEPG medium is paralleled by the lack of inhibition of growth on YEPG solid medium even when concentrations of 10.0 γ/ml or more of oligomycin are used.

In view of the specificity of inhibition of oligomycin and the correlation between its inhibitory effects in liquid and solid culture, a series of mutants resistant to this inhibitor were isolated.

Isolation of the oligomycin resistant mutants

The parental strains D6 and D22 which were oligomycin sensitive were

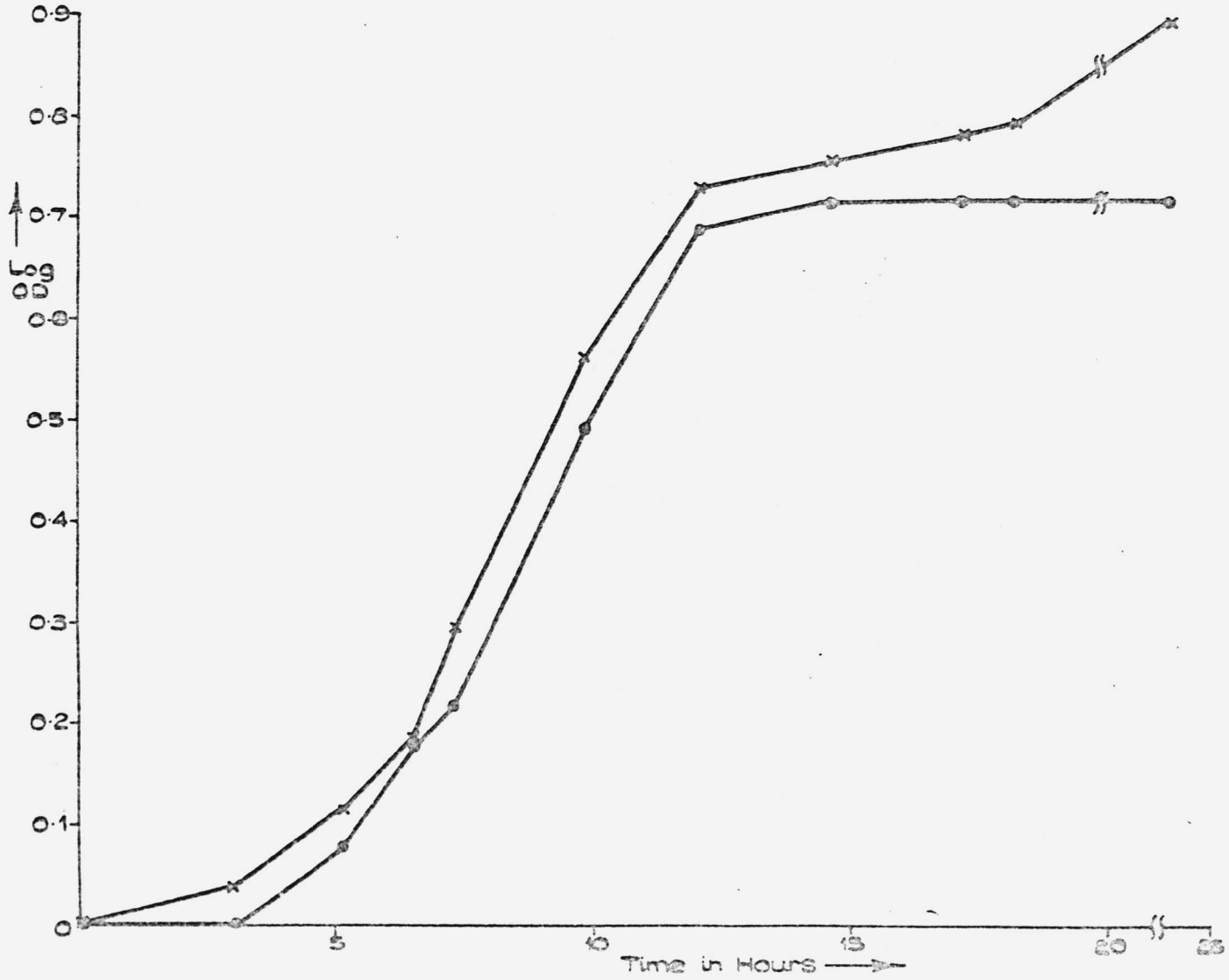
LEGEND TO FIGURE 4.3.

Inhibitory Effects of Oligomycin

2 ml of a D22 starter culture (YEPG stationary phase) were inoculated into 100 ml aliquots of YEPG contained in 500 ml sidearm flasks. Either 5.0 γ/ml oligomycin in ethanolic solution or an equivalent amount of ethanol (0.2%) was added to the flasks immediately following this inoculation. Growth was followed using an EEL colorimeter fitted with a 607 filter. The cultures were grown at 30^{o}C.

Key

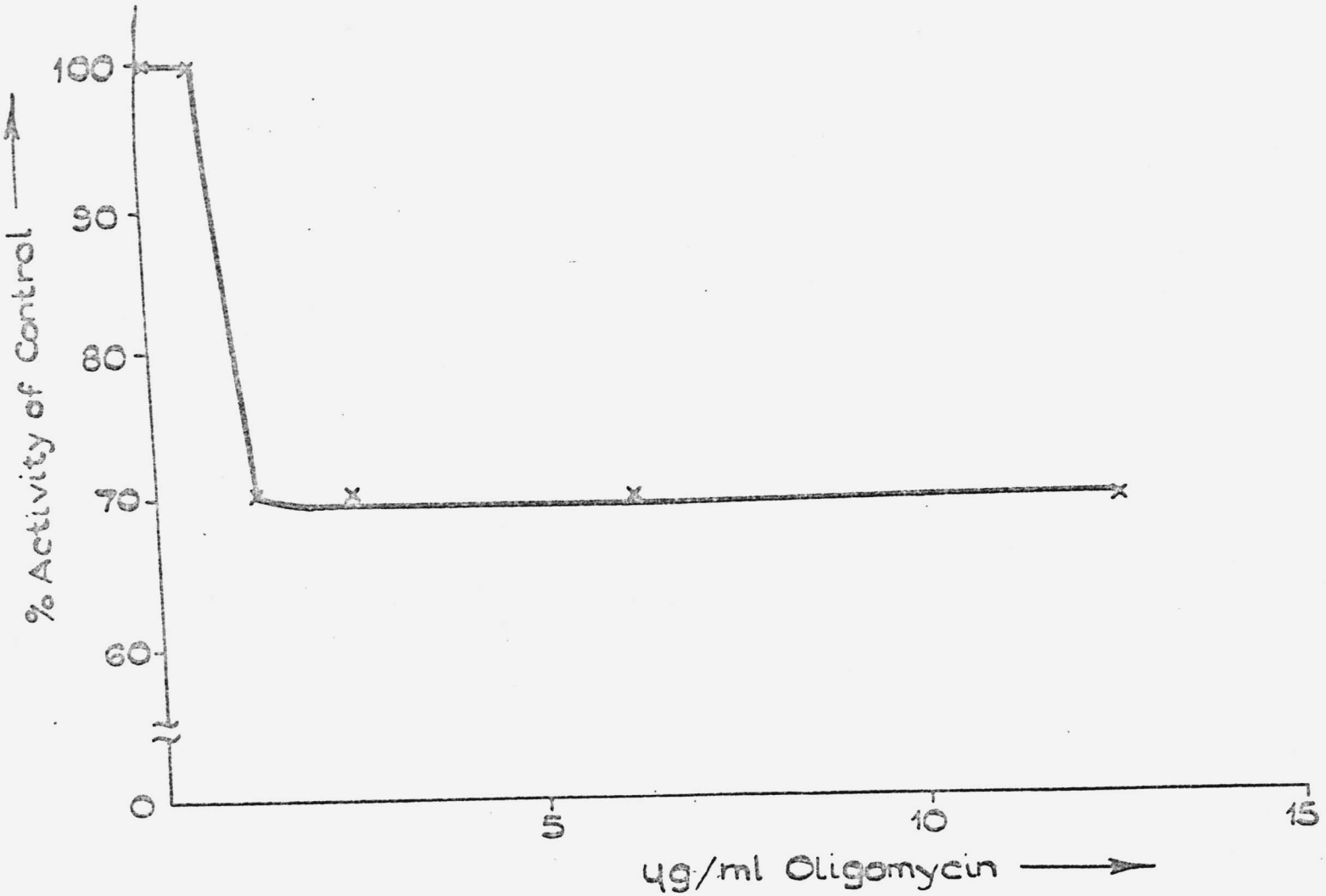
- • + 5.0 γ/ml oligomycin
- × + EtOH


0·9
0·8
0·7
0·6
0·5
0·4
0·3
0·2
0·1
0
Log OD
5
10
15
20
25
Time in Hours

LEGEND TO FIGURE 4.4.

Inhibitory Effects of Oligomycin.

1% inocula of the starter culture (stationary phase YEPG) were added to 100 ml of YEPG in 500 ml sidearm flasks which were grown at 30°C for 48 hours on an orbital shaker. Dry weights were then taken. Oligomycin was added to the flasks as an ethanolic solution (ethanol concentrations equalised) prior to the addition of the inocula.


100
90
80
70
60
0
% Activity of Control
5
10
15
µg/ml Oligomycin

grown on YEPG liquid medium until they had reached stationary phase, when they were u.v. irradiated for 80 seconds using the standardised procedure described in chapter 2. The irradiated suspension was spread undiluted on to freshly prepared oligomycin YEPGly plates using 0.2 ml of this suspension per plate. In view of the results described in the preceding section, plates containing 2.5, 5.0 and 10.0 γ/ml of oligomycin were used for mutant isolation. The plates were incubated at 30^{o}C and inspected at regular intervals for growth. The primary resistant colonies only appeared slowly, generally becoming noticeable on the plates only after 4 - 5 days incubation. After 7 - 8 days, some of these resistant colonies were picked off, streaked on to YEPG plates and the resistance of single reisolated colonies checked using 1.25 and 2.5 γ/ml oligomycin YEPGly plates. Those isolates that were still resistant after two such purification steps were stocked. In total, 63 resistant strains were originally isolated - 45 of them derived from D22 and 18 from D6. Table 4.1. shows the details of the mutant isolation procedure.

It was found in agreement with Parker et al (Parker et al., 1968) that the oligomycin resistant mutants occur with fairly high frequency. The isolation procedure used by these workers, however, differed from that described here in some particulars and generally resulted in the isolation of mutants exhibiting lower levels of oligomycin resistance. The use of an 80 second irradiation period resulting in 14 and 10% cell viability for D22 and D6 respectively should have minimised the possibility of multiple gene mutations - a point that will be returned to later. It can be seen from Table 4.1. that the frequency of resistant mutants isolated declined as the oligomycin concentration in the isolating plate increased. All of the 63 mutants except 3 have remained completely stable since their isolation. The strains that have altered have increased their level of oligomycin resistance; in no case has resistance been lost.

Characterisation of the levels of resistance to Oligomycin and Rutamycin of the Oligomycin Resistant mutants

Following purification, the level of oligomycin resistance of the mutant

TABLE 4.1.

Details of the Isolation Procedure for Oligomycin Resistant Mutants

TABLE 4.1.

	D22			D6		
	γ/ml oligomycin in isolation plates			γ/ml oligomycin in isolation plates		
	2.5	5.0	10.0	2.5	5.0	10.0
Total number of OL^R colonies isolated	22	21	4	12	-	3
Density of cells in u.v. irradiated suspension	2×10^7/ml	2×10^7/ml	2×10^7/ml	$\sim 2 \times 10^7$/ml	$\sim 2 \times 10^7$/ml	$\sim 2 \times 10^7$/ml
% Viability of plated cells after irradiation	14.0	14.0	14.0	10.0	10.0	10.0
% OL^R colonies of total cells plated	5.5×10^{-4}	5.5×10^{-4}	1.0×10^{-4}	3.0×10^{-4}	-	7.5×10^{-5}
% OL^R colonies of total viable cells plated	3.9×10^{-3}	3.9×10^{-3}	7.1×10^{-4}	3.0×10^{-3}	-	7.5×10^{-4}

strains was examined by dropping out cell suspensions on to oligomycin YEPGly plates. The results are presented in Table 4.2. The majority of the strains were resistant to over 10.0 γ/ml oligomycin, regardless of whether they were isolated on 2.5, 5.0 or 10.0 γ/ml oligomycin drug plates. Within the 'A' series of mutants isolated from drug plates containing 2.5 γ/ml oligomycin, 4 out of the 21 resistant mutants derived from parental strain D22 were resistant to 2.5 γ/ml oligomycin and 2 resistant to 5.0 γ/ml oligomycin. The rest were resistant to over 10.0 γ/ml oligomycin. Within the 'B' series of D22 derived mutants isolated on 5.0 γ/ml oligomycin drug plates, 2 of the 19 mutants are resistant to 5.0 γ/ml oligomycin, 1 to 2.5 γ/ml oligomycin and the remainder to over 10.0 γ/ml. All the 'C' series derived from D22 on 10.0 γ/ml oligomycin plates are resistant to greater than 10.0 γ/ml oligomycin. The absence of any sizeable number of mutants resistant to levels of oligomycin below the level of oligomycin present initially in the isolation plates, indicates the stability of the oligomycin within the plating medium. This result reflecting the stability of oligomycin to breakdown is in agreement with other findings reported by Lardy (Lardy et al., 1964) and Smith (Smith et al., 1954). Because of the expense involved, screening of the mutants with concentrations of oligomycin greater than 10.0 γ/ml was omitted.

The results of screening the oligomycin resistant mutants for resistance to rutamycin at concentrations of up to 100.0 γ/ml are also shown in Table 4.2. All of the oligomycin resistant mutants possess increased resistance to rutamycin. Where it is possible to state a definite upper resistance limit for both oligomycin and rutamycin, rutamycin appears to be as efficient, or at least 50% as efficient as oligomycin in inhibiting the growth of the strains tested. This relationship holds with both the parental strains D6 and D22 and the resistant strains isolated from them. The presence of resistance to rutamycin in all the oligomycin resistant mutants, the similarities in the growth inhibitory doses to both drugs, the constant correlation between increased resistance to oligomycin and rutamycin in the mutants, as well as the similarities in their action on mutants where the oligomycin resistance is thermosensitive (chapter 5), all suggest the equivalence of these drugs in vivo. This is in agreement with studies on mitochondria in vitro (Lardy et al., 1965). The

TABLE 4.2.

Oligomycin and Rutamycin Resistance of the Resistant Mutants

Oligomycin and rutamycin resistances were assayed by dropping out cells onto YEPGly plates containing

0, 1.25, 2.5, 5.0 and 10.0 γ/ml oligomycin and

0, 2.5, 5.0, 10.0, 50.0 and 100.0 γ/ml rutamycin.

Plates were incubated for 3 days at 30^{o}C prior to scoring.

TABLE 4.2.

Strain	Oligomycin Resistance γ/ml (1)	Rutamycin Resistance γ/ml (2)	Ratio (1)/(2)
D22	0.5	1.0 - 2.0	1:2 - 1:4
D22A1	2.5	5.0	1:2
D22A2	> 10.0	50.0	≤ 1:5
D22A3	> 10.0	50.0	≤ 1:5
D22A4	> 10.0	25.0	≤ 1:3
D22A5	> 10.0	> 100.0	-
D22A6	> 10.0	N.T.	-
D22A7	> 10.0	100.0	-
D22A8	> 10.0	N.T.	-
D22A9	2.5 - 5.0	5.0 - 10.0	1:2
D22A11	2.5	5.0	1:2
D22A12	> 10.0	100.0	-
D22A13	> 10.0	25.0 - 50.0	-
D22A14	> 10.0	50.0	-
D22A15	> 10.0	25.0 - 50.0	-
D22A16	> 10.0	> 100.0	-
D22A18	2.5	5.0	1:2
D22A19	> 10.0	> 100.0	-
D22A20	5.0 - 10.0	25.0 - 50.0	1:3
D22A21	> 10.0	> 10.0	-
D22A22	> 10.0	25.0 - 50.0	-
D22B1	> 10.0	> 100.0	-
D22B2	> 10.0	N.T.	-
D22B3	> 10.0	> 100.0	-
D22B4	> 10.0	> 50.0	-
D22B5	> 10.0	N.T.	-
D22B6	> 10.0	> 100.0	-
D22B8	> 10.0	25.0	≤ 1:2
D22B9	> 10.0	N.T.	-
D22B10	2.5 - 5.0	5.0 - 10.0	1:2
D22B11	5.0	N.T.	-
D22B13	10.0	N.T.	-
D22B15	> 10.0	100.0	-
D22B16	> 10.0	25.0 - 50.0	-
D22B19	> 10.0	N.T.	-
D22B20	5.0	5.0 - 10.0	1:1 - 1:2
D22B21	> 10.0	> 100.0	-
D22B22	2.5	5.0 - 10.0	1:2 - 1:4
D22B23	10.0	> 10.0	-

TABLE 4.2.

Strain	Oligomycin Resistance γ/ml (1)	Rutamycin Resistance γ/ml (2)	Ratio (1)/(2)
D22C1	> 10.0	> 100.0	-
D22C2	> 10.0	> 100.0	-
D22C4	> 10.0	> 100.0	-
D6	0.5 - 0.75	< 2.5	1 :2 - 1 :4
D6A1	> 10.0	N.T.	-
D6A3	2.5	5.0	1 :2
D6A4	> 10.0	N.T.	-
D6A5	> 10.0	> 100.0	-
D6A8	2.5 - 5.0	2.5	1 :1 - 1 :2
D6A9	> 10.0	50.0	-
D6A10	2.5	5.0 - 10.0	1 :2 - 1 :4
D6A11	2.5	2.5 - 5.0	1 :1 - 1 :2
D6A15	1.25 - 2.5	5.0	1 :2 - 1 :4
D6B1	> 10.0	> 100.0	-
D6B2	> 10.0	100.0	-
D6B3	> 10.0	50.0	-

failure to observe oligomycin resistance without resistance to rutamycin is in disagreement with the report of Stuart (Stuart, 1970). Stuart found that some 4% of the oligomycin resistant mutants isolated were of this type. In view of the low frequency of occurrence of this type of mutant, our failure to find such mutants may simply be due to sampling error.

Stuart also found that the ratio of oligomycin resistance to rutamycin resistance varied considerably, not only amongst the resistant mutants but also amongst the parental strains used. This ratio varied from 1 :2 for strain 351 to 1 :20 in the case of strains 22 and DV147. Again, such results have not been found in these experiments.

There is no obvious correlation between the level of resistance of the mutant to rutamycin and oligomycin and the parental strain from which it was derived. In the case of the majority of mutants, there is at least a twenty-fold increase in resistance to oligomycin associated with a fifty- to hundred-fold increase in rutamycin resistance. The existence of such large changes in resistance, together with the known high specificity of these drugs, provides some reassurance that the resistance of these mutants are not due to small changes in cell permeability, but may be due to changes at the actual locus of action of the drug.

Cross resistance studies with Antimycin A, Chloramphenicol, Mikamycin, Erythromycin and Spiramycin

The specificity of the oligomycin resistant mutants was first assessed by testing their resistance to a variety of other agents affecting mitochondrial metabolism. The drugs used - chloramphenicol, erythromycin, mikamycin, antimycin A and spiramycin - were chosen because they affect mitochondrial activity but not, at least primarily, oxidative phosphorylation. Two of the drugs, chloramphenicol and mikamycin belonging respectively to the chloramphenicol and streptogramin groups of antibiotics (Vasquez et al., 1969), are inhibitors of mitochondrial protein synthesis (Linnane and Haslam, 1970) and specifically inhibit protein synthesis occurring on 70s ribosomes, the site of inhibition actually being on the 50s subunit (Vasquez et al., 1969). Both these agents are reported to interfere with mitochondrial electron transport although at higher concentrations (Dixon et al., 1971 ; Freeman, 1970 ;

Hanson and Hodges, 1963). Erythromycin and spiramycin, which are also 70s protein synthesis inhibitors, are both members of the macrolide antibiotic group as is oligomycin. These two drugs have not been implicated as having any subsidiary direct effect on electron transport (Vasquez et al., 1969). Antimycin A is a highly specific electron transport inhibitor, blocking the electron transport chain between cytochrome b and cytochrome c_1 (Kaniuga et al., 1969; Potter and Reif, 1952). It has not been implicated as having any effect on mitochondrial protein synthesis and indeed, its mode of action as a fungicide which is without effect on bacterial growth, argues strongly against such a secondary site of action (Leben and Keitt, 1948).

Table 4.3. shows the concentrations of the various inhibitors necessary to inhibit glycerol and glucose supported growth of the wild type strains D22 and D6. There is clearly a specific effect of these agents on mitochondrial metabolism at concentrations where general cellular metabolism is unaffected. In Table 4.4. the results of the cross resistance screening are listed. The oligomycin resistant mutants can be subdivided into two major classes on the basis of their cross resistance to the four inhibitors shown in Table 4.4. and erythromycin (not shown). Mutants placed in class I show cross resistance to all the drugs tested. Mutants placed in class II on the other hand, fail to show cross resistance to any of them. The class II mutants, if anything, actually appear to have even lower resistance to these drugs than the parental strains, D6 and D22. The magnitude of this effect, though slight, was especially noticeable when the assays with chloramphenicol were scored.

Although a general rise in resistance to all these drugs is typical of the class I mutants, there appears to be no correlation between the level of primary resistance to oligomycin and rutamycin of a particular mutant and the cross resistance shown to these four drugs (compare : D22 A2 vs. D22 A9; D22 B20 vs. D22 A9 and D22 A11 vs. D22 A9). In all cases the levels of cross resistance shown are low - some two- to four-fold greater than those of the parental strains and of a different order of magnitude from the primary resistances to oligomycin and rutamycin. The level of resistance to rutamycin of the mutants is frequently some 50 - 100 times greater than that of the parental strains, whilst the cross resistance shown by the class I mutants to these four drugs is as already stated, only two- to four-fold. The exact increase in cross resistance to each of the four drugs exhibited by a particular class I mutant is not necessarily identical, but the

TABLE 4.3.

Inhibitory Levels of Various Drugs on YEPG and YEPGly Media

Drugs were added to YEPG and YEPGly media, as solid in the case of chloramphenicol but otherwise as ethanolic solutions. Strains D22 and D6 were tested for their resistance to these drugs by dropping out and the plates scored after 3 days' incubation at 30°C. The drug concentrations used were :

Antimycin A, 0.005, 0.01, 0.05, 0.5, 2.0, 5.0 and 10.0 γ/ml

Chloramphenicol, 0.5, 1.0, 2.0 and 4.0 mg/ml

Erythromycin, 0.5, 1.0, 2.0 and 5.0 mg/ml

Mikamycin, 0.005, 0.01, 0.05 and 0.3 mg/ml

Rutamycin, 1.0, 2.0, 10.0, 50.0 and 250.0 γ/ml

Spiramycin, 0.1, 0.5, 1.0, 2.0 and 4.0 mg/ml

TABLE 4.3.

Compound	Strain			
	D6		D22	
	Glucose	Glycerol	Glucose	Glycerol
Antimycin A	10 0 γ/ml	< 0.005 γ/ml	10 0 γ/ml	< 0.005 γ/ml
Chloramphenicol	> 4.0 mg/ml	1.0 mg/ml	> 4.0 mg/ml	0.5 mg/ml
Erythromycin	> 5.0 mg/ml	< 0.5 mg/ml	> 5.0 mg/ml	< 0.5 mg/ml
Mikamycin	> 1.0 mg/ml	< 0.005 mg/ml	> 1.0 mg/ml	< 0.005 mg/ml
Spiramycin	-	0.5 - 1.0 mg/ml	-	< 0.5 mg/ml
Rutamycin	250.0 γ/ml	1.0 - 2.0 γ/ml	-	1.0 - 2.0 γ/ml

TABLE 4.4.

Cross Resistance Studies to 'Mitochondrial' Inhibitors

Mikamycin resistance was tested using 0.005, 0.01, 0.05 and 0.3 mg/ml mikamycin in YEPGly medium.

Chloramphenicol resistance was tested using 0.5, 1.0, 2.0 and 4.0 mg/ml chloramphenicol in YEPGly medium.

Spiramycin resistance was tested using 0.1, 0.5, 1.0, 2.0 and 4.0 mg/ml spiramycin in YEPGly medium.

Oligomycin resistance was tested using 0.5, 1.0, 2.5, 5.0 and 10.0 γ/ml oligomycin in YEPGly medium.

Antimycin A resistance was tested using 0.005, 0.01, 0.05, 0.5, 2.0, 5.0 and 10.0 γ/ml antimycin A in YEPGly medium.

Cells of the various strains were dropped out onto these drug plates as well as control YEPGly plates and incubated at 30°C for 3 days prior to scoring.

TABLE 4.4.

Strain	Classes I and III				
	Oligomycin Resistance γ/ml	CAP Resistance mg/ml	Spiramycin Resistance mg/ml	Mikamycin Resistance mg /ml	Antimycin A Resistance γ/ml
D22 (OL^S_S)	0.5	0.5	< 0,5	< 0.005	< 0.005
D6 (OL^S)	0.7	1.0	0.5 - 1.0	< 0.005	< 0.005
D22 A1 (III)	2.5	< 1.0	0.1 - 0.5	0.01	0.005
D22 A2	2.5	2.0	0.5	0.01	0.01
D22 A3	> 10.0	1.0	0.5	0.01	0.01
D22 A4	> 10.0	2.0	1.0	0.01	0.01
D22 A5	> 10.0	2.0	0.5	0.005	N.T.
D22 A7	> 10.0	2.0	2.0	0.01	0.01
D22 A9	2.5 - 5.0	1.0	0.5	0.01	0.01
D22 A11	2.5	2.0	1.0	0.01	0.01
D22 A12	> 10.0	2.0	1.0	N.T.	N.T.
D22 A20	5.0 - 10.0	2.0	1.0	0.005	0.01
D22 B3	> 10.0	2.0	2.0	0.05	0.01
D22 B4	> 10.0	2.0	0.5	0.01	0.01
D22 B6	> 10.0	2.0	1.0 - 2.0	0.01	0.01
D22 B8	> 10.0	2.0	0.5 - 1.0	0.01	0.01
D22 B9	> 10.0	2.0	0.5	0.01	0.01
D22 B10	2.5 - 5.0	1.0	0.5	0.01	0.01
D22 B15	> 10.0	1.0	N.T.	0.01	0.01
D22 B16	> 10.0	2.0	1.0	0.01	0.01
D22 B20	5.0	2.0	1.0	0.01	0.005
D22 B22	2.5	1.0	0.5	0.01	0.01
D22 C1	> 10.0	2.0	2.0	0.05	0.01
D6 A3	2.5	1.0 - 2.0	2.0	0.01	0.01
D6 A4	> 10.0	2.0	2.0	0.01	0.01
D6 A9	> 10.0	2.0	2.0	0.01	0.01
D6 A10	2.5	2.0	2.0	0.01	0.01
D6 A11	2.5	2.0	2.0	0.01	0.01
D6 A15	1.25 - 2.5	2.0	N.T.	0.01	0.01
Control Strain					
D22 Er310	0.5	< 1.0	N.T.	< 0.005	N.T.

TABLE 4.4.

Strain	Class II				
	Oligomycin Resistance γ/ml	CAP Resistance mg/ml	Spiramycin Resistance mg/ml	Mikamycin Resistance mg/ml	Antimycin A Resistance γ/ml
D22A13	> 10.0	< 0.5	0.1	< 0.005	< 0.005
D22A14	> 10.0	< 0.5	0.1 - 0.5	< 0.005	< 0.005
D22A15	> 10.0	< 0.5	0.5	< 0.005	< 0.005
D22A16	> 10.0	< 1.0	0.1	< 0.005	< 0.005
D22A18	2.5	< 0.5	0.1	< 0.005	< 0.005
D22A19	> 10.0	< 0.5	0.1	< 0.005	< 0.005
D22A21	> 10.0	< 0.5	0.1	< 0.005	< 0.005
D22B1	> 10.0	< 0.5	0.1	< 0.005	< 0.005
D22B21	> 10.0	< 0.5	0.1	< 0.005	< 0.005
D22B23	10.0	< 0.5	N.T.	< 0.005	< 0.005
D22C2	> 10.0	< 1.0	0.1	< 0.005	< 0.005
D22C4	> 10.0	< 0.5	0.1	< 0.005	< 0.005
D6A1	> 10.0	< 1.0	0.1	< 0.005	N.T.
D6A5	> 10.0	< 1.0	0.5	< 0.005	< 0.005
D6B1	> 10.0	< 1.0	0.1 - 0.5	< 0.005	< 0.005
D6B2	> 10.0	< 1.0	0.1	< 0.005	< 0.005
D6B3	> 10.0	-	0.1	< 0.005	< 0.005

significance of these variations is difficult to assess. Despite these slight variations in cross resistance shown by many of the strains, D22 A1 provides the only exception to the neat classification into two classes, cross resistance in this strain to mikamycin and antimycin A being completely unaccompanied by resistance to the other two inhibitors tested, chloramphenicol and spiramycin. For this reason D22 A1 has been placed in a class of its own - class III.

A number of other mutants have been screened in order to assess whether the cross resistance observed are a phenomena restricted to oligomycin resistant strains. None of the other strains tested, which have included two erythromycin resistant mutants, D22 Er 310 and D22 Er 510, showing cytoplasmic inheritance, a spiramycin resistant strain, D22 Sp 2 :1, also showing cytoplasmic inheritance and a double erythromycin cycloheximide resistant mutant, D22 Er 310 cyclo 1 :2, showed cross resistance to any of the four drugs used in this section. Similar tests on a large number of chloro CCP resistant mutants also failed to show the presence of cross resistance in these strains

Cross Resistance Studies to Uncoupling Agents

Table 4.5. shows the concentrations of TTFB, chl CCP, DNP and octyl DNP needed to inhibit the growth of the wild type yeast strains D6 and D22 when glucose and glycerol are the carbon sources as well as the concentrations of these agents needed to affect metabolic functions of chloroplasts and mitochondria in vitro.

The concentrations of chl CCP required to inhibit the glycerol supported growth of strains D6 and D22 agrees closely with those concentrations producing a stimulation of oxygen uptake in intact yeast cells, and a similar correlation is observable for TTFB. In both cases, however, the concentrations required are much greater than those required to uncouple mammalian mitochondria in vitro. Similarly, with DNP, there is a close correlation between the concentration of DNP needed to inhibit glycerol supported growth and that leading to a stimulation of oxygen uptake in intact yeast cells, once the effects of pH have been taken into account, it being known that DNP enters the intact yeast cell only in the free acid form (Field et al., 1934 ; unpublished information).

In the case of these three uncouplers as well as octyl DNP, lower concentrations were found necessary to inhibit glycerol supported yeast growth than is necessary for inhibition of growth on glucose medium. It is therefore apparent

TABLE 4.5.

Effect of the Uncouplers DNP, Octyl DNP, Chloro CCP and TTFB on Various Metabolic Functions

Metabolic Function	Effective Concentration of Uncoupler			
	DNP	Octyl DNP	Chloro CCP	TTFB
Stimulation of ATPase	2.1×10^{-4}M (3)	2.3×10^{-6}M (3)	-	2.4×10^{-8}M (4)
Uncoupling of Mammalian Mitochondria	5.0×10^{-5}M (1)	-	$1.0 - 10.0 \times 10^{-7}$M (7)	3.0×10^{-8}M (4)
Uncoupling of Photo-phosphorylation	3.3×10^{-4} (2) $- 1 \times 10^{-3}$M	-	1.6×10^{-6} (7) $- 5.0 \times 10^{-6}$M	$1.0 - 2.0 \times 10^{-6}$M (5)
Stimulation of QO_2 in Intact Yeast Cells	$1.0 - 2.0 \times 10^{-5}$M (free acid)	-	$1.0 - 5.0 \times 10^{-5}$M	$4.0 - 5.0 \times 10^{-5}$M
Inhibition of Growth on YEPGly	1.0×10^{-5}M (free acid)	$1.0 - 2.0 \times 10^{-5}$M (free acid)	$1.0 - 2.5 \times 10^{-5}$M	$4.0 - 5.0 \times 10^{-5}$M
Inhibition of Growth on YEPG	$25.0 - 5.0 \times 10^{-5}$M (free acid)	$25.0 - 5.0 \times 10^{-5}$M (free acid)	$1.0 - 2.0 \times 10^{-4}$M	3.0×10^{-4}M

NOTE : All values for yeast are for D22

Source of Reference

(1) Losada and Arnon (1963)
(2) Neumann and Jagendorf (1964)
(3) Slater (1963)
(4) Beechey (1966)
(5) Buchel et al. (1965)
(6) Kempson (1971)
(7) Heytler (1963)

Other values given were determined by the author

that these agents retain some degree of specificity for mitochondrial metabolism in vivo. The observation that both DNP and octyl DNP inhibit glycerol and glucose supported growth at similar concentrations, despite the higher lipid solubility of octyl DNP (much less octyl DNP than DNP being necessary for maximal stimulation of rat liver mitochondrial ATPase in vitro (Slater, 1963)), suggests that the lipid solubility of the uncoupler is not the limiting step in vivo.

The results of the cross resistance studies to TTFB and chloro CCP are shown in Table 4.6. Those mutants which, on the basis of their cross resistance to antimycin A, chloramphenicol, etc. were designated class I, similarly show increased levels of resistance to TTFB and chloro CCP compared with the wild type strains. The class II mutants, whether derived from D6 or D22, similarly show a level of resistance as low, or generally lower, than the parental strain from which they were derived - a result also obtained in the cross resistance studies previously described. This is perhaps best demonstrated by the TTFB results obtained with strains D6 A1, D6 B1, D22 B1 and D22 A13. No correlation was again apparent between the level of cross resistance to the uncouplers TTFB and chloro CCP and the level of resistance to rutamycin and oligomycin - even amongst the class I mutants. The increase in resistance among the class I mutants is again small, being only two- to four-fold. That the resistance is low does not seem to be due to the small differential between the specific mitochondrial and non-specific general cellular inhibition produced by uncouplers. Table 4.5. indicates that for both chloro CCP and TTFB, the differential between these two levels of interaction is some six- to ten-fold, well above the resistance increases noted with the class I mutants. Few of these strains, for instance, are resistant to more than 30 γ/ml of TTFB or 5×10^{-5}M chloro CCP and yet glucose supported growth of D22 and D6 can tolerate concentrations of up to 50 γ/ml and $1 - 2 \times 10^{-4}$M of TTFB and chloro CCP respectively without showing any inhibition.

These low levels of cross resistance are in accord with the level of cross resistances to antimycin A, etc. reported in the previous section where the differential sensitivity between the non-specific and specific effects of the inhibitors is much greater (see Table 4.3) than for the uncouplers. The increased resistance to TTFB shown by the class I mutants when growth is glycerol supported is not matched by a similar increase in resistance when glucose is the

TABLE 4.6.

Strain	Classes I and III Mutants		
	Oligomycin Resistance γ/ml	chl CCP Resistance X 10^{-5} M	TTFB Resistance γ/ml
D22 (OL^S_S)	0.5	1.0	8.25
D6 (OL^S)	0.7	1.0	2.5
D22A1 (III)	2.5	2.5	20.0
D22A2	2.5	2.5	35.0
D22A3	> 10.0	5.0	30.0
D22A4	> 10.0	5.0	20.0
D22A5	> 10.0	N.T.	35.0
D22A7	> 10.0	5.0	35.0
D22A9	2.5 - 5.0	2.5	30.0 - 35.0
D22A11	2.5	N.T.	20.0
D22A12	> 10.0	5.0	35.0
D22A20	5.0 - 10.0	N.T.	20.0
D22B3	> 10.0	2.5 - 5.0	30.0
D22B4	> 10.0	N.T.	N.T.
D22B6	> 10.0	2.5	30.0
D22B8	> 10.0	5.0	15.0
D22B9	> 10.0	5.0	25.0 - 30.0
D22B10	2.5 - 5.0	N.T.	25.0 - 30.0
D22B15	> 10.0	5.0	35.0
D22B16	> 10.0	2.5	30.0 - 35.0
D22B20	5.0	N.T.	20.0
D22B22	2.5	N.T.	20.0
D22C1	> 10.0	2.5	35.0
D6A3	2.5	N.T.	N.T.
D6A4	> 10.0	5.0	10.0 - 15.0
D6A9	> 10.0	5.0	10.0
D6A10	2.5	N.T.	8.25
D6A11	2.5	N.T.	5.0
D6A15	1.25 - 2.5	N.T.	N.T.
Control Strains			
D22ER110	0.5	N.T.	10.0
D22ER310	0.5	1.0	5.0
D22CP514	0.5	5.0	30.0 - 35.0
D22CP714	0.5	5.0	35.0
D22CP1814	0.5	5.0	35.0

TABLE 4.6.

Strain	Class II Mutants		
	Oligomycin Resistance γ/ml	chl CCP Resistance X 10^{-5} M	TTFB Resistance γ/ml
D22 A13	> 10.0	1.0	5.0
D22 A14	> 10.0	N.T.	2.5
D22 A15	> 10.0	N.T.	5.0
D22 A16	> 10.0	1.0	5.0
D22 A18	2.5	1.0	< 10.0
D22 A19	> 10.0	N.T.	5.0
D22 A21	> 10.0	N.T.	5.0
D22 A22	> 10.0	N.T.	5.0
D22 B1	> 10.0	N.T.	5.0
D22 B21	> 10.0	2.5 - 5.0	5.0
D22 B23	10.0	N.T.	N.T.
D22 C2	> 10.0	N.T.	5.0 - 8.25
D22 C4	> 10.0	N.T.	5.0
D6 A1	> 10.0	5.0	< 2.5
D6 A5	> 10.0	N.T.	N.T.
D6 B1	> 10.0	5.0	< 2.5
D6 B2	> 10.0	N.T.	N.T.
D6 B3	> 10.0	N.T.	< 2.5

energy source. This strongly suggests that the increased cross resistances shown by the class I mutants to TTFB and therefore, by implication, to all the other drugs to which cross resistance is shown, is not due to a cell permeability change or a general cell detoxifying phenomena as in this case both the glucose and glycerol supported growth should show comparable increases in resistance. No cross resistance to either DNP or octyl DNP was shown by any of the oligomycin resistant mutants, whether of class I or class II when the assays were carried out in media adjusted originally to pH 5.8 (Table 4.7). A similar failure to demonstrate cross resistance to these two uncouplers occurred when the assay was repeated using media adjusted to pH 3.5. A similar observation that increased resistance to chloro CCP and TTFB is not accompanied by an increased tolerance to DNP and octyl DNP has been made in the case of the chloro CCP resistant mutants (unpublished information).

Cross Resistance Studies with
Triethyltin, 1799 and Cycloheximide

Both 1799 and triethyltin inhibit the growth of strains such as D22 and D6 on YEPGly at concentrations where growth on YEPG is unaffected. Both the parental strains are, for instance, inhibited by 0.5 - 1.0 γ/ml triethyltin when growing on YEPGly, whilst on YEPG, inhibition does not occur in the case of D22 until between 20.0 - 40.0 γ/ml is present and in the case of D6, until 40.0 γ/ml of triethyltin is added. Similarly, approximately 5.0 γ/ml of 1799 inhibits the glycerol supported growth of these wild type strains whilst over 200 γ/ml have been added to glucose media without any inhibition of their growth being apparent. 1799 appears to differ from the other uncouplers as the concentration differential between its specific mitochondrial mode of action and its non-specific inhibition of whole cell metabolism is much greater than is observed with chloro CCP, TTFB and DNP. The concentration differential of 1799 is at least thirty-fold whilst the other uncouplers show maximally a concentration differential of eight- to ten-fold. This apparent dissimilarity between 1799 and the other uncouplers utilised is also apparent in the cross resistance studies of triethyltin mutants which have been performed by Mr. W. Lancashire (private information). Comparison of the concentration of 1799 necessary to uncouple oxidative phosphorylation of rat liver and beef heart mitochondria *in vitro* with that required to inhibit glycerol supported yeast growth, shows that a five- to forty-fold greater

TABLE 4.7.

Cross Resistance to DNP and Octyl DNP

Strains were dropped out onto unbuffered YEPGly plates containing various concentrations of DNP or octyl DNP.

The concentrations of DNP used were
3.75×10^{-4}M, 2.5×10^{-4}M, 1×10^{-4}M, 7.5×10^{-5}M,
5×10^{-5}M, 2.0×10^{-5}M, 1×10^{-5}M.

The concentrations of octyl DNP used were
5×10^{-4}M, 1×10^{-4}M, 7.5×10^{-5}M, 5×10^{-5}M,
2.5×10^{-5}M, 1×10^{-5}M.

Scoring was performed after 3 days' incubation at 30^{o}C.

TABLE 4.7.

Strain	Class	DNP Resistance pH 5.8	Octyl DNP Resistance pH 5.8
D22	-	2×10^{-4}M	$1 - 5 \times 10^{-4}$M
D22 A4	I	2×10^{-4}M	$1 - 5 \times 10^{-4}$M
D22 A13	II	2×10^{-4}M	$1 - 5 \times 10^{-4}$M
D22 B1	II	2×10^{-4}M	$1 - 5 \times 10^{-4}$M
D22 B9	I	2×10^{-4}M	$1 - 5 \times 10^{-4}$M
D22 B16	I	2×10^{-4}M	$1 - 5 \times 10^{-4}$M
D22 Er 310	-	2×10^{-4}M	$1 - 5 \times 10^{-4}$M

concentration is required for inhibition in the latter case (Heytler, 1970). In the case of triethyltin, concentrations of between 3.0×10^{-2}M and 1.0×10^{-7}M inhibit rat liver mitochondrial oxidative phosphorylation (Aldridge and Street, 1964), compared to the $2.0 - 4.0 \times 10^{-6}$M necessary to inhibit glycerol supported growth of the wild type strains.

Table 4.8 shows the results of the cross resistance screening. The only strains showing cross resistance are those which have already been designated as class I on the basis of their cross resistance to other drugs. The class II mutants again show, if anything, less resistance to both these drugs than the parental strain from which they were derived. Only D22A1 shows some inconsistencies, being non-resistant to triethyltin whilst showing increased resistance to 1799. This strain has already been shown to be slightly anomalous and placed in class III itself on the basis of its resistance to the protein synthesis inhibitors (Table 4.4). The cross resistance shown by the class I oligomycin resistant mutants to 1799 and triethyltin differs from that seen with the other drugs tested in that it involves increases in resistance, often of ten-fold or more. Although this response is still not comparable to that shown towards rutamycin (compare D22A7 for instance), it is considerably greater than that shown towards any of the protein synthesis inhibitors or other uncouplers - and in the former case, at least the response is not limited by non-specific cellular inhibition.

Table 4.9. shows the results of screening various other types of mutant to 1799. None of the cytoplasmic erythromycin resistant mutants show any resistance to this drug and few of the chloro CCP resistant strains show resistance and none to high levels. The only mutant other than the class I oligomycin resistant strains to show such behaviour is a double mutant known to be carrying cytoplasmically inherited erythromycin resistance and a cycloheximide resistance determinant of which the mode of inheritance is unknown. As the original $Er^{R}210$ strain from which this double mutant was spontaneously isolated does not show this cross resistance to 1799, the resistance factor must be suspected of being isogenic with the cycloheximide resistance determinant. Because of this result preliminary screening of the class I and class II mutants to cycloheximide has been carried out. When concentrations of around 1.0 γ/ml are used, the same pattern of cross resistance classification is seen with this drug as with all the

TABLE 4.8.

Cross Resistance Studies with Triethyltin and '1799'

Cells of the various strains were dropped out onto YEPGly plates containing 0, 2.5, 5.0, 12.5, 17.5, 25.0 and 50.0 γ/ml of '1799' and triethyltin plates containing 0, 0.25, 0.5, 1.0, 2.5, 5.0 and 10.0 γ/ml of triethyltin. Plates were scored after 3 days incubation at 30°C.

TABLE 4.8.

Strain	Classes I and III Mutants		
	Oligomycin Resistance γ/ml	Triethyltin Resistance γ/ml	'1799' Resistance γ/ml
D22 (OL^S_S)	0.5	0.5	2.5 - 5.0
D6 (OL^S_S)	0.7	0.5	5.0
D22 A1 (III)	2.5	0.5	17.5
D22 A2	2.5	10.0	> 50.0
D22 A3	> 10.0	5.0	> 50.0
D22 A4	> 10.0	5.0 - 10.0	> 50.0
D22 A5	> 10.0	5.0	> 50.0
D22 A7	> 10.0	10.0	> 50.0
D22 A9	2.5 - 5.0	5.0	25.0
D22 A11	2.5	10.0	> 50.0
D22 A12	> 10.0	10.0	> 50.0
D22 A20	5.0 - 10.0	5.0	25.0
D22 B3	> 10.0	5.0 - 10.0	> 50.0
D22 B4	> 10.0	N.T.	N.T.
D22 B6	> 10.0	10.0	> 50.0
D22 B8	> 10.0	5.0	> 50.0
D22 B9	> 10.0	5.0	> 50.0
D22 B10	2.5 - 5.0	5.0	N.T.
D22 B15	> 10.0	10.0	> 50.0
D22 B16	> 10.0	5.0 - 10.0	> 50.0
D22 B20	5.0	5.0	25.0
D22 B22	2.5	5.0	25.0
D22 C1	> 10.0	10.0	-
D6 A3	2.5	N.T.	N.T.
D6 A4	> 10.0	2.5	25.0
D6 A9	> 10.0	5.0	25.0
D6 A10	2.5	2.5	25.0
D6 A11	2.5	2.5	25.0
D6 A15	1.25 - 2.5	N.T.	2.5 - 5.0

TABLE 4.8.

Strain	Class II Mutants		
	Oligomycin Resistance γ/ml	Triethyltin Resistance γ/ml	'1799' Resistance γ/ml
D22 (OL^S_S)	0.5	0.5	2.5 - 5.0
D6 (OL^S_S)	0.7	0.5	5.0
D22A13	> 10.0	0.5	2.5
D22A14	> 10.0	0.5	2.5
D22A15	> 10.0	0.5	5.0
D22A16	> 10.0	0.5	2.5
D22A18	2.5	0.5	2.5
D22A19	> 10.0	0.5	2.5 - 5.0
D22A21	> 10.0	0.5	2.5
D22A22	> 10.0	0.5	2.5
D22B1	> 10.0	0.5	2.5
D22B21	> 10.0	0.5	N.T.
D22B23	10.0	N.T.	2.5 - 5.0
D22C2	> 10.0	0.5	2.5
D22C4	> 10.0	0.5	2.5
D6A1	> 5.0	< 0.5	5.0
D6A5	> 10.0	-	5.0
D6B1	> 10.0	0.5	5.0
D6B2	> 10.0	N.T.	N.T.
D6B3	> 10.0	< 0.5	2.5

TABLE 4.9.

Screening of Control Strains to '1799'

The strains were dropped out onto YEPGly plates containing 0, 2.5, 5.0, 12.5, 25.0 and 40.0 γ/ml of 1799

Plates were scored after 3 days' incubation at 30°C

TABLE 4.9.

Strain	Genotype	Concentrations of '1799' γ/ml					
		0	2.5	5.0	12.5	25.0	40.0
D22	$Er^{S}OL^{S}CP^{S}Cy^{S}$	+	+	-	-	-	-
D22 Er 210	$Er^{R}OL^{S}CP^{S}Cy^{S}$	+	+	-	-	-	-
D22 Er 310	$Er^{R}OL^{S}CP^{S}Cy^{S}$	+	+	-	-	-	-
D22 Er 510	$Er^{R}OL^{S}CP^{S}Cy^{S}$	+	+	-	-	-	-
D22 Er 210 Cy 1 :2	$Er^{R}OL^{S}CP^{S}Cy^{R}$	+	+	+	+	+	+
D22 CP 914	$Er^{S}OL^{S}CP^{R}Cy^{S}$	+	+	+	-	-	-
D22 CP 1114	$Er^{S}OL^{S}CP^{R}Cy^{S}$	+	+	+	-	-	-

others used, the class I mutants showing cross resistance and the class II mutants apparently none. These results are not interpreted as showing that there is a cell permeability barrier change in the class I mutants, both because of the biochemical data of J. R. Turner (private communication ; in press 1972) which demonstrates that the mitochondria are oligomycin resistant and because of the data already quoted showing no change in TTFB resistance in the presence of glucose.

These observations may be explicable by postulating that there is a common factor to the mitochondrion and the cellular function(s) affected by cycloheximide. As the inhibitory action of cycloheximide has been shown to involve inhibition of GTP hydrolysis by the soluble TF2 factor, it may be that this GTPase and the mitochondrial ATP synthetase involve a shared factor(s) (Traub, 1969). The recent report that cycloheximide inhibits only membrane-bound ribosomes and is ineffective against free ribosomes suggests that alterations in a membrane component could equally well be responsible (Glazer and Sartorelli, 1972) for the cross resistance if the mutation causes changes in all cellular membranes.

Cross resistance studies to aurovertin

When both the parental strains D6 and D22 and the oligomycin resistant mutants derived from them were tested for resistance to aurovertin by dropping out cells onto YEPGly plates to which aurovertin had been added, no growth inhibition was observed even at concentrations of 10.0 γ/ml. Aurovertin is therefore markedly less effective than oligomycin in suppressing glycerol supported yeast growth whether the comparison is made on a molar concentration or weight basis. This difference in effectiveness is at least ten- to twenty-fold. This result contrasts with the findings of Lardy et al. who observed that oligomycin and aurovertin were equally effective in inhibiting in vitro oxidative phosphorylation of rat liver mitochondria (Lardy et al., 1964). There is the possibility that the Lardy et al. results are due to the use of superoptimal amounts of oligomycin. However, comparison of the data of Lardy et al. concerning aurovertin with the data of Huijing and Slater who studied the inhibition by oligomycin of rat liver mitochondrial oxidative phosphorylation using similar mitochondrial protein concentrations in their assays to those used by Lardy et al. suggests that maximally only a four- to six-fold difference in sensitivity to

aurovertin and oligomycin can exist in vitro (Lardy et al., 1964; Huijing and Slater, 1969). Even this estimate assumes that the concentration of aurovertin used in the experiments of Lardy et al. was not similarly superoptimal. Lee and Ernster have reported that aurovertin is, if anything, more effective than oligomycin in inhibiting the phosphorylation of sub-mitochondrial particles when NADH is used as the respiratory substrate and equally effective in inhibiting the ATP-P_i exchange reaction (Lee and Ernster, 1968).

The disparity between the in vitro results observed when assaying mitochondria for oxidative phosphorylation and the results observed in vivo with yeast could be due to

(i) An instability of aurovertin compared to oligomycin in the plating media

(ii) Difference in the ease of entry into the yeast cell of the two compounds

(iii) Differences in sensitivity of yeast and mammalian mitochondria to these two inhibitors, all the in vitro results being extrapolated from studies on mammalian mitochondria. Differences between yeast and mammalian mitochondria are known to exist (Onishi, 1970).

(iv) Oligomycin may have key effects on growth which aurovertin lacks. It is known that in vitro oligomycin affects activities connected with the energy conservation process unaffected by aurovertin (Lardy et al., 1964; Connelly and Lardy, 1964).

Due to the scarcity of aurovertin it was impracticable to test resistance against higher levels of aurovertin by the usual method of 'dropping out.' Testing of some selected strains for their resistance to aurovertin was therefore performed using well diffusion plates (Table 4.10).

Both D6 and D22 were inhibited by aurovertin on YEPGly plates using these high aurovertin concentrations whilst no inhibition of D22 was apparent when the aurovertin was added to the strain on a YEPG plate. The failure of Baldwin et al. to find inhibition of Candida albicans when concentrations of up to 1.0 mg/ml of aurovertin were used was probably therefore due to the presence of fermentable substrate in the test medium as this yeast, like S.cerevisiae, is a strong fermenter of both glucose and maltose. Table 4.10. shows also that all the class I mutants tested were all cross resistant to aurovertin whilst

TABLE 4.10.

Cross Resistance to Aurovertin

Overnight grown YEPG cultures of the various strains were diluted and 700 - 1000 cells spread onto YEPGly plates which were dried before central wells were cut. 0.1 or 0.2 ml of a fresh 1.0 mg/ml ethanolic aurovertin solution was added to each plate. Control plates contained 0.2 ml of ethanol in the central well. Plates were scored after incubation for 2 - 3 days at 30°C.

TABLE 4.10.

Strain	Mutant Class	Resistance to Oligomycin γ/ml	Inhibition by 200γ Aurovertin	Inhibition by 100γ Aurovertin	Ethanol Control Inhibition
D22	(OL^S)	0.5	+	+	-
D6	(OL^S)	0.75	+	N.T.	-
D6 A4	I	> 10.0	-	-	-
D22 A2	I	2.5	-	-	-
D22 A4	I	> 10.0	-	-	-
D22 A5	I	> 10.0	-	-	-
D22 B15	I	> 10.0	-	-	-
D22 B16	I	> 10.0	-	-	-
D22 A13	II	> 10.0	+	N.T.	-
D22 A21	II	> 10.0	+	N.T.	-
D22 C4	II	> 10.0	+	N.T.	-
D22 CP514	(CP^R)	0.5	+	N.T.	-

none of the class II mutants tested or the control strain, a chloro CCP resistant mutant, showed any resistance. It is therefore apparent that the cross resistance behaviour already shown by the class I mutants towards other drugs is repeated with aurovertin. The presence of a class of mutants resistant to oligomycin but not to aurovertin does, in view of the biochemical analysis of these mutants, support the non-identity of the sites of inhibition of oligomycin and aurovertin.

Cross resistance to DCCD

In view of both the known mode of action of DCCD in inhibiting ATP synthesis associated with mitochondrial electron transport (Beechey et al., 1967) (for other references see chapter 1) and that DCCD is capable of immediately inhibiting oxygen uptake on addition to intact yeast cells (Kovác et al., 1968b) (chapter 7 of this thesis), cross resistance studies to this drug have been carried out.

As DCCD and other carbodiimides are well known carboxyl group activating reagents and have been shown to react covalently with the functional groups of proteins such as -COOH, $-NH_2$, -SH, serine -OH, tyr -OH (Abrams and Baron, 1970), the cross resistance studies were initially carried out using a synthetic medium (Wickerhams) supplemented with only the adenine, arginine and methionine required for the growth of the auxotrophic yeast strains, rather than the usual complex medium. The results of preliminary experiments carried out with buffered medium at pH 4.6 and 7.3 are shown in Table 4.11. The concentrations of DCCD required to inhibit both glycerol and glucose supported growth are much higher at pH 4.6 than 7.3 - thus glucose supported growth of strain D22 at pH 4.6 is present even when 500μM DCCD was used, whilst at pH 7.3, only 125μM DCCD caused cessation of glucose supported growth. This effect is presumably due to both the difference in pH of the growth medium, together with the effect of the difference in buffering ion. The latter factor may also account for the discrepancy between these results and those of Kovác et al. (Kovác et al., 1968b) who found that 500μM DCCD inhibited glucose supported growth in a phosphate buffered medium at pH 4.6. The absence of any data as to the length of the growing period in Kovác's experiments, however, makes comparisons difficult. Table 4.11. also demonstrates that at both pH 4.6 and 7.3, the concentration of DCCD required to inhibit glycerol supported growth is much

TABLE 4.11.

Inhibitory Concentration of DCCD for Glucose vs. Glycerol Supported Growth

Drug plates were made up in minimal medium supplemented with adenine, arginine and methionine in either 0.1 M acetate buffer, pH 4.6 or 0.1 M phosphate buffer, pH 7.3. Plates were used within 12 hours of preparation.

The concentrations of DCCD used were 0, 12.5, 25.0, 50.0, 125 and 500 μM. Plates were scored after 7 days' incubation at 30^{0}C.

TABLE 4.11.

	pH 4.6		pH 7.3	
	D22	D6	D22	D6
Glycerol	25.0 - 50.0 μM	25.0 - 50.0 μM	< 12.5 μM	< 12.5 μM
Glucose	> 500 μM	> 500 μM	125 μM	-

less than that required when glucose is the carbon source, the differential being at least ten-fold. These results suggest that DCCD, despite its reactivity, appear capable of acting in a specifically mitochondrial mode, even when incorporated into a plating medium.

The results of a cross resistance study on oligomycin resistant mutants is shown in Table 4.12. Most of the oligomycin resistant strains showed some slight increase in resistance to DCCD compared with the wild type strain. This was generally not more than two- to four-fold and a strict class I : class II correlation such as has been demonstrated in the other cross resistance studies is not apparent. This result with D22 showing greater DCCD sensitivity than the majority of the mutants, has been obtained in several repeat experiments, though the exact concentration of DCCD required for inhibition was markedly variable. Any attempt to give a more definite interpretation of these cross resistance studies is complicated by the very poor growth of many of the strains at pH 7.3 when glycerol rather than glucose is the carbon source. Attempts to improve the resolution of the experiment by using ethanol instead of glycerol as the non-fermentable carbon source at the same pH were useless as it did not support growth at all.

Other attempts using complex media and synthetic media extensively supplemented with amino acids and nucleotides were all unsuccessful when carried out at pH 7.3 as no growth inhibition was seen at the DCCD concentrations tried. When the cross resistance studies were repeated on synthetic medium supplemented only with arginine, methionine and adenine but buffered at pH 6.0 (another attempt to overcome this problem) an entirely different cross resistance to that shown at pH 7.3 was apparent, and there was no obvious correlation between oligomycin resistance and the level of resistance to DCCD. Indeed, all that can be said is that D6, D22 and the class II mutants have apparently a higher level of resistance than that shown by most of the class I mutants.

These results are most easily interpretable in two ways :

(i) DCCD breakdowns or complexes with the plating medium, and this breakdown is pH dependent. The inhibition of yeast growth would then be due to both/either DCCD and/or the breakdown product

TABLE 4.12.

Cross Resistance Studies with DCCD

Plates of minimal medium supplemented with adenine, arginine and methionine were buffered with 0.1 M phosphate buffer, pH 7.2. Plates were used within 12 hours of pouring. The concentrations of DCCD used were 0, 0.5, 1.0, 2.5 and 5.0 μM. Plates were scored after 7 days incubation at 30^{o}C.

TABLE 4.12.

Strain	Oligomycin Resistance γ/ml	DCCD Resistance μM	Class
D22	0.5	< 0.5	
D22 A8	N.T.	< 0.5	
D22 A13	> 10.0	2.5 - 5.0	II
D22 A16	> 10.0	0.5	II
D22 B7	N.T.	1.0	
D22 B8	> 10.0	1.0	I
D22 B10	2.5 - 5.0	5.0	I
D22 B14	N.T.	1.0	
D22 B16	> 10.0	1.0 - 2.5	I
D22 B17	N.T.	0.5 - 1.0	
D22 B21	> 10.0	1.0	II
D22 B22	2.5	1.0	I
D22 B23	10.0	1.0	II
D22 C4	> 10.0	< 0.5	II
D6 A8	2.5 - 5.0	1.0	

and this is responsible for the change in the cross resistance pattern.

(ii) DCCD reacts at several sites within the cell and within the mitochondrion and the site of reaction is markedly pH dependent. There is some evidence for DCCD having multiple sites of action within the intact yeast cell (Kovác et al., 1968b).

In view of the known reactivity of DCCD and the failure to obtain quantitatively reproducible results even at the same pH, the first alternative seems more apposite.

Further studies were discontinued because of these complications and the limited information produced from these experiments.

Discussion

The results presented in this chapter show the very high specificity of oligomycin and rutamycin as inhibitors of mitochondrial metabolism. The 250 γ/ml of rutamycin which fails to inhibit glucose supported yeast growth and therefore does not affect general cellular metabolism, is at least 250 times greater than that required to inhibit glycerol supported growth. In contrast, none of the uncouplers, except 1799, have greater than a ten-fold difference in their specific mitochondrial and non-specific modes of action. Of the agents tested, only antimycin A, 1799 and some of the protein synthesis inhibitors approach the degree of specificity of oligomycin. The failure of Smith et al. (Smith et al., 1954) in the original publication on oligomycin, to find growth inhibition by oligomycin of S.cerevisiae, was presumably due to the presence of fermentable substrate in all three of the media used. Yeast extract glucose agar, potato agar, bacto wort agar will all contain either maltose or glucose. Whilst this explanation for the lack of inhibition of S.cerevisiae will suffice for it and also for the lack of inhibition of H.anomala and Endomyces vernalis which are respectively strong and weak fermenting yeasts, it probably does not account for the lack of inhibition of Cryptococcus neoformans which ferments neither glucose nor maltose.

Although the results obtained with oligomycin define the mitochondrion as the site of action in vivo, they do not, and indeed cannot by their very nature, define its mode of action within the mitochondria. As oligomycin is a macrolide antibiotic (Parker et al., 1968) belonging to the same antibiotic group as erythromycin and spiramycin and as both Parker et al., and Kovác et al.

(Kovác et al., 1970) have shown that it alters the cytochrome content of glucose grown yeast cells, lowering the amounts of cytochrome b and cytochrome c, and completely abolishing the presence of cytochromes $a + a_3$, theoretically it was possible that oligomycin in vivo might be acting as a 70s ribosomal protein synthesis inhibitor. This is, however, unlikely as oligomycin does not inhibit the growth of either gram positive or gram negative bacteria (Smith et al., 1954). This behaviour of oligomycin is quite unlike that of erythromycin and spiramycin (Vasquez, 1967; Hahn, 1967) which strongly inhibit in vivo mainly gram positive bacteria, but are also active at higher concentration against some gram negative bacteria. The presence of glucose in the test media used by Smith et al. (Smith et al., 1954) would, of course, make no difference to the validity of the tests on bacteria, if oligomycin was acting as a protein synthesis inhibitor. Furthermore, Bronk (Bronk, 1963) has shown that ^{14}C leucine incorporation into rat liver mitochondria in vitro is not affected by oligomycin applied at the same concentration as inhibits oxidative phosphorylation. Even a ten- to forty-fold increase of oligomycin above this level only reduced the ^{14}C leucine incorporation by 30 - 40%. As the leucine incorporation was dependent on mitochondrial energy conservation (no added ATP), even this small inhibition is explicable on the basis of oligomycin affecting energy transfer. This latter evidence is not definitive as Firkin and Linnane (Firkin and Linnane, 1969) have shown that whilst chloramphenicol and erythromycin were effective inhibitors of ^{14}C leucine incorporation of yeast mitochondria in vivo, only the former was effective with rat liver mitochondria. However, oligomycin also fails to inhibit the respiratory adaptation of anaerobically grown yeast (Kovác et al., 1970). The small inhibition of respiration caused by oligomycin is largely relievable by CCP and therefore presumably reflects oxygen uptake reduced by the blocking of coupled oxidation rather than any interference with protein synthesis (Kovác et al., 1970). It should be emphasised that these experiments were carried out in the presence of high concentrations of oligomycin (20 γ/ml).

Other evidence suggesting that the decrease in cytochrome levels in oligomycin treated cells is not a primary effect but an indirect one which oligomycin has in common with such other diverse mitochondrial agents such as antimycin A, DNP, chloro CCP and cyanide, has been reported by Lusikov et al. who showed

that all these agents, in the absence of cell growth, caused decreases in the levels of NADH oxidase, cytochrome c oxidase and succinate oxidase activity measured in the intact yeast cell (Lusikov et al., 1970). The many reports of oligomycin inhibition of both respiration and energy linked processes in intact cells in essentially short term experiments, also clearly suggest that the in vivo and in vitro inhibition sites of oligomycin are identical (Kovác et al., 1970; Minakami et al., 1963; Dallner and Ernster, 1962; Tobin and Slater, 1965; Currie and Gregg, 1965; Harary and Slater, 1965).

Parker et al. (Parker et al., 1968) have considered two explanations of the action of oligomycin on mitochondrial cytochrome levels in the intact yeast cell. Firstly, that it acts by complexing with a 'lattice' or structural protein, thus masking it and causing a failure of mitochondrial self-assembly. Secondly, it acts by affecting the level of mitochondrial ATP and thus affects mitochondrial protein synthesis and cytochrome levels. The former alternative seems to imply a large structural change and seems unlikely in view of the presence of most of the cytochromes excluding cytochromes $a + a_3$ in oligomycin treated cells - albeit in reduced quantities. The findings of Ycas (Ycas, 1956), Bartley and Tustanoff (Bartley and Tustanoff, 1966) and Kovác et al. (Kovác et al., 1970) that antimycin A affects mitochondrial cytochrome levels similarly to oligomycin and the aforementioned findings of Lusikov et al. (Lusikov et al., 1970) suggest the second alternative may be more tenable. The effect, even if mediated by changes in the ATP pool levels, may not, however, be due only to an effect on protein synthesis. For instance, the speed of the onset of respiratory chain degradation observed by Lusikov et al. in the absence of cell division would imply a very considerable turnover of mitochondrial protein if the only site of action of agents such as chloro CCP and oligomycin was on protein synthesis (Lusikov et al., 1970). In this respect the finding by Estrada-O et al. (Estrada-O et al., 1964) that certain soluble proteins - glutamic dehydrogenase and glutamic aspartic transaminase but not cytochrome c - are released from mitochondria incubated in vitro with 2:4 DNP, oligomycin and triamcinolone may be important, especially as none of the agents other than triamcinolone apparently caused mitochondrial swelling in the incubation buffer used (Estrada-O, 1964). The implication that factors other than swelling are important in this process is reinforced by the finding that the enzyme loss caused by inorganic phosphate is reversed by some phospholipids without affecting the degree of mitochondrial swelling (Estrada-O et al., 1970).

Neither this decrease of cytochrome levels in oligomycin treated non-resistant yeast strains nor the growth inhibition which is apparent on non-fermentable substrates is due to petite induction caused by oligomycin. Although in these experiments it has been found, in agreement with Parker et al. (Parker et al., 1968) but in disagreement with Kovác et al. (Kovác et al., 1970) that oligomycin along with many other agents (Nagai et al., 1961) increase the petite frequency, the increase was only three- to five-fold respectively for D6 and D22 (see also chapter 7), and this is insufficient to account for either the observed drop in cytochrome levels, or the growth inhibition.

The results of the cross resistance studies on the oligomycin resistant mutants show quite clearly that the mutants fall, with one exception, into two categories, the first group, class I, being resistant to antimycin A, mikamycin, chloramphenicol, erythromycin, spiramycin, TTFB, 1799, CCP, triethyltin and aurovertin, as well as rutamycin and oligomycin, whilst the second class are resistant only to rutamycin and oligomycin. For all the agents except 1799 and triethyltin, the increase in these secondary resistances is small - three- to five fold as against an increase in the primary resistance to oligomycin and rutamycin of, in many cases, a hundred-fold. In the case of triethyltin, the level of resistance was often increased by ten- to twenty-fold, whilst in the case of 1799, resistance increases of at least twenty-fold were obtained. The high level of resistance increase to 1799 is not understood as it stands in opposition to the other uncouplers tested such as TTFB and chloro CCP. This difference may well be related to the greatly increased specificity that 1799 shows for mitochondrial metabolism compared with the other uncouplers and is also reflected in the different behaviour it shows towards both triethyltin mutants (W. Lancashire, private communication) and chloro CCP resistant mutants in comparison to chloro CCP and TTFB (unpublished information).

The class I mutants are not regarded as multigenic mutants as :

(i) they were isolated after a comparatively short u.v. irradiation period - 14 and 10% of the D22 and D6 irradiated cells respectively were viable after the irradiation treatment.

(ii) If the mutants are to be considered multigenic, then over 50% of the mutants isolated must be multigenic as over 50% of the mutants isolated are of the class I

type. This appears unlikely.

(iii) Sporulation of diploids obtained from crossing class I mutants by a sensitive tester has failed to separate the cross resistances from the primary oligomycin resistance. Only oligomycin sensitive haploids without the associated cross resistance or oligomycin resistance haploids showing the cross resistances were obtained on sporulating diploids of this type.

It therefore seems probable that the class I mutants are single gene mutants, the genetic determinant causing extensive pleiotropic effects. Cytoplasmically determined mutants apparently showing pleiotropic effects have been isolated by Bunn et al. (Bunn et al., 1970).

The failure to find cross resistance in the class I mutants originally (Avner and Griffiths, 1970) was due to the fact that 1799, which gives higher levels of increased resistance relative to the wild type, was not available in the initial screening, and the levels of the other drugs used were too high to detect these low levels of cross resistance. Figure 4.5. illustrates diagramatically how resistance to oligomycin and other drugs affecting mitochondrial metabolism might develop. This resistance could be :

(i) Due to the cell wall or plasma membranes.

(ii) Due to cytoplasmic detoxifying agents.

(iii) Due to mitochondrial permeability barriers.

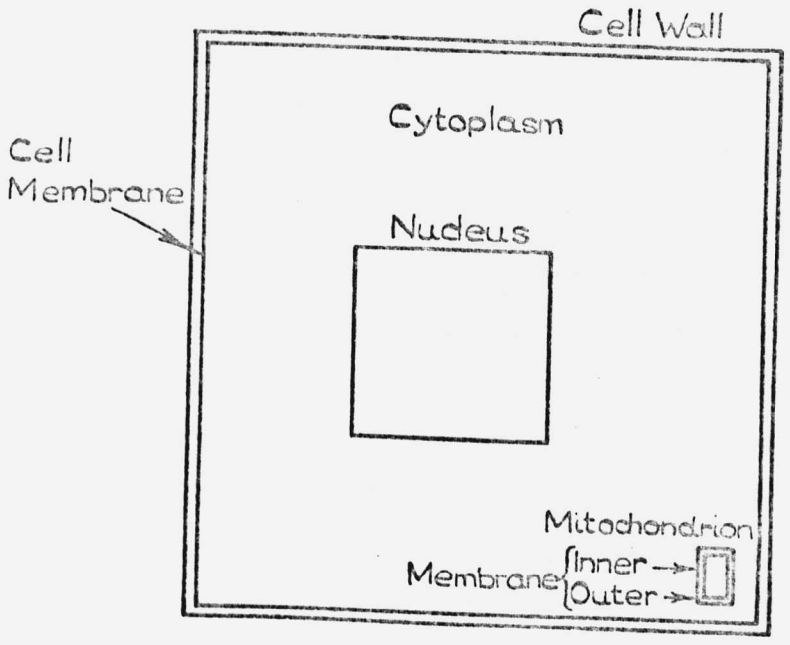
(iv) Due to mitochondrial detoxifying agents, including binding phenomena.

(v) Due to alterations in the primary site of action of the drug.

The finding that the genetic determinant(s) conferring the class II type of resistance is located on the mitochondrial DNA (chapter 6) suggests that the resistance of these mutants is likely to be due to changes in the mitochondria itself. Similarly, the presence in the class I mutants of cross resistance to such a variety of drugs affecting mitochondrial metabolism suggests that in the class of mutant also the resistance is likely to be localised at the mitochondrial level. As the drugs to which cross resistance is shown possess little similarity other than their lipophilicity, it seems unlikely that drug detoxification is responsible for the phenotypes of the class I mutants.

J. R. Turner (private communication) and Griffiths et al. (Griffiths et al., 1972)

Figure 4.5. Schematic Diagram of Possible Sites of OL Resistance.



in an extensive series of biochemical studies of these mutants, have shown that the ATPase activity of mitochondria and submitochondrial particles from both class I and class II mutants shows, in comparison with mitochondria from the wild type strains D6 and D22, increased levels of resistance to oligomycin inhibition. The class I mutant mitochondria however, unlike those of the class II mutants examined, tend to lose their resistance on storage at $0^{o}C$, so that the ATPase activity becomes progressively more sensitive to oligomycin with time. The resistance to oligomycin of both classes of mutants does, however, seem to be due to changes in the mitochondria itself. As increased levels of resistance are also found in submitochondrial particles, the resistance is not, moreover, due to changes in mitochondrial permeability to oligomycin. Analysis of the results reveals however, that the situation is less clear cut for the class I mutants than is apparent in the analysis of the class II mutants, particular class I mutants which show high resistance in vivo showing comparatively slight resistance in in vitro assays.

Components of the mitochondria which might be responsible for the changes in oligomycin sensitivity of the mitochondria and mitochondrial ATPase include not only the ATPase complex itself, but due to the allotopic effects characteristic of the oligomycin sensitivity of the ATPase, many of the components of the mitochondrial inner membrane. Both protein and lipid factors may be implicated. The proteins will include the 'OSCP' which appears to be synonomous with the tripartite stalk visible on negative staining and to which the F_1 is attached (MacLennan and Asai, 1968). Electron microscopic analysis of some oligomycin resistant mutants has, however, shown that these mutants appear to have a normal mitochondrial morphology as observed by negative staining, and to have retained the 'OSCP' (Linnane, in press). It is perhaps naive to expect gross morphological changes in these mutants, such as deletions of proteins, which might be visible by electron microscopy when the selection procedure used ensures all the mutants isolated must have functional oxidative phosphorylation systems. This is not to doubt however, that point mutations affecting the 'OSCP' may be conferring the cellular oligomycin resistance.

A number of observations suggests that the oligomycin resistance of the class II mutants which show cytoplasmic inheritance (chapter 6) is unlikely to be

due to changes in either the F_1 or OSCP proteins. For instance, both the F_1 and OSCP have been reported to be synthesised on cytoplasmic ribosomes (Tzagaloff, 1969; Tzagaloff, 1970). In addition, ATPase activity has been demonstrated in petite strains, albeit in reduced amounts. In contrast to the ATPase activity from grande strains however, this ATPase activity is oligomycin insensitive (Schatz, 1968). The interpretation of these results is, however, open to various objections such as, that cytoplasmic translation may not imply nuclear or non-mitochondrial coding and that as the ATPase appears to be a large complex (Catterall and Pedersen, 1971; Senior and Brooks, 1970), it is impossible to dismiss the hypothesis that it is part mitochondrial and part nuclear coded. In addition, there is no evidence as to whether the two recombination groups governing the class II resistance phenotype serve a regulatory or structural function.

Tzagaloff has recently reported finding four proteins which are made on mitochondrial ribosomes and apparently concerned in conferring oligomycin sensitivity on the mitochondrial ATPase (Tzagaloff, in press; Tzagaloff, 1972). These proteins, which are found when rutamycin sensitive ATPase complexes are extracted from mitochondria, but not when rutamycin insensitive ATPase complexes are prepared, are obvious possibilities for the locus of oligomycin resistance in the class II mutants.

The possibility that changes in the lipid moiety of the mitochondrial membrane could confer increased oligomycin resistance is supported by the findings of Palatini and Bruni (Palatini and Bruni, 1970) that certain phospholipids reverse oligomycin inhibition of mitochondria in vitro. Similarly, the finding by Kagawa and Racker (Kagawa and Racker, 1966) that phospholipids are necessary to restore oligomycin sensitive ATPase activity to isolate an F_1 fraction when added back to a CF_o fraction, implicates phospholipids as having an important role in determining the sensitivity or otherwise of the mitochondrial ATPase towards oligomycin. Linnane's group have also shown the great sensitivity of the yeast mitochondrion and oxidative phosphorylation to alterations in lipid composition by work on the KD 115 mutant which is unable to synthesise unsaturated fatty acids. Mutants of this type, when deprived of exogeneous fatty acids, rapidly lose their oxidative phosphorylative capacity, even though they are capable of growing if a fermentable energy supply is provided (Proudlock et al., 1969; Linnane and Haslam, 1970).

Mutations causing pleiotropic effects so characteristic of the class I mutants may be rationalised within a membrane system, on the basis of the lipoprotein protomer subunit structure of such systems (Green and Perdue, 1966). Depending on its universality, changes in a single membrane component, whether lipid or protein, may result, either through allotopism alone or through a combination of allotopic and allosteric interactions, in the alteration of a large number of different metabolic activities.

Thus, Conner et al. have shown that a change in the predominant triterpene alcohol of T. pyriformis from tetrahymenol to ergosterol results in a large decrease in resistance to a large number of antibiotics such as filipicin and nystatin which affect membranes by attacking sterol : phospholipid interactions (Conner et al., 1971). This effect seems also to be present in isolated membranes. Evidence suggesting that changes in the phospholipid moiety of the membrane could cause the cross resistance changes seen in the class I mutants is not restricted solely to the report by Palatini and Bruni (Palatini and Bruni, 1970) that oligomycin inhibition in vitro is preventable and reversible by phospholipids but is also provided by a variety of other reports showing similar effects of phospholipids on the other drugs to which the class I mutants show cross resistance. Thus, Bruni and coworkers (Bruni et al., 1971) have also reported the effectiveness of some phospholipids in reversing DCCD inhibition whilst Aldridge has stressed the affinity of triethyltin for particular phospholipids (Aldridge and Street, 1964). McGivan (McGivan and Chappell, 1967) found that cardiolipin increases the resistance of lipoprotein micelles to anion entry and since oxidative phosphorylation uncouplers generally are weak acids and have been postulated to enter the mitochondria as anions (Van Dam and Slater, 1967), such behaviour, if repeated in the intact mitochondria, could lead to increased resistance to uncouplers. The postulate is also in agreement with the results of Weinbach et al. who found more pentachlorophenol bound to lipid deficient mitochondria than to intact mitochondria (Weinbach and Garbus, 1965). These results suggest, therefore, that changes in the lipid, or more specifically, the phospholipid composition of the mitochondrion could result in simultaneously changed resistance to the uncouplers, DCCD and oligomycin. As, moreover, Moore and Umbreit have reported that the synthetic activity of ribosomes from S. fecalis is markedly increased by the presence of membrane, and their incorporation

of amino acids is proportional to phospholipid concentration (Moore and Umbreit, 1964; Moore and Umbreit, 1965), it does not appear too far-fetched to suggest that alteration of phospholipids, either quantitatively, or qualitatively, might also affect slightly the resistance to antibiotics affecting protein synthesis.

It is possible that many of the reports in the literature showing effects of phospholipids on inhibitors, for instance DCCD and oligomycin inhibition (Palatini and Bruni, 1970; Bruni et al., 1971) may simply be due to partition effects on the predominantly lipophilic inhibitors. Such a mode of action in vivo would, however, account well for the small increases in cross resistance found in the class I mutants. As already mentioned, it is, however, quite conceivable that the cross resistances are caused by a direct change in a protein rather than a lipid component of the mitochondrion. Such a hypothesis derives largely from the theory that there is some sort of extensive 'structural' protein(s) element in the mitochondrion which, because of the protein : protein interactions inherent to membrane systems, is capable of causing changed behaviour in many mitochondrial metabolic activities simultaneously. Woodward and coworkers (Woodward and Munkres, 1966; Woodward, 1968; Munkres and Woodward, 1966; Woodward and Munkres, 1967) have produced some evidence to support this idea. For instance, the enzymic parameters characteristic of both malate dehydrogenase and fumarate reductase are greatly affected by binding to a mitochondrial 'structural' protein preparation. Furthermore, substitution of structural protein preparations from the cytoplasmic Neurospora mutants mi-1 and mi-3 instead of the wild type, caused large changes in the enzymic parameters measured, suggesting exactly the sort of situation envisaged above regarding the class I mutants. In respect of such a hypothesis, it is interesting that many uncoupling agents will bind to mitochondrial membrane fractions (Weinbach and Garbus, 1965) or 'structural' protein preparations (Zalkin and Racker, 1965).

It is, moreover, possible to envisage a system where any protein occurring extensively enough in the mitochondrion might cause similar interactions and effects, so there need not be a restriction such as that the altered protein must be 'structural' and non-catalytic. In view of the present uncertainty whether there is any such thing as a 'non-catalytic structural protein', whether there are families of such proteins, whether they are high molecular proteins or low

molecular polypeptides (Laico et al., 1970), whether 'structural protein' preparations just contain denaturated catalytic membrane proteins (Schatz and Saltzgaber, 1969), or whether the so-called 'structural protein(s)' are a specialised class of highly hydrophobic catalytic proteins, this latter possibility should not be overlooked.

Further analysis of the class I mutants utilising the temperature sensitive mutants of this class will hopefully allow greater understanding of the underlying biochemical lesion(s).

CHAPTER 5

PHENOTYPIC SCREENING II - TEMPERATURE SENSITIVITY OF OLIGOMYCIN RESISTANT MUTANTS

Introduction

Temperature sensitive mutants affected in a variety of metabolic activities have been isolated in both procaryotic and eucaryotic organisms. In the former the work of Edgar and his collaborators on T_4 phage provides an example of the power of this technique (Edgar and Lielausis, 1964; Epstein et al., 1963). Temperature sensitive mutants amongst eucaryotic organisms have been obtained recently in Chlamydomonas reinhardi (McMahon, 1971) whilst extensive series of such mutants in S.cerevisiae have been accumulated by Hartwell and his associates (Hartwell, 1967; Hartwell and McLaughlin, 1968a; Hartwell and McLaughlin, 1968b; Hartwell et al., 1970). These workers have isolated five classes of temperature sensitive mutants. Three of the classes of mutants have lesions respectively in either DNA, RNA or protein synthesis, whilst the other two classes of mutants appear to have lesions in energy metabolism and membrane composition. Additional classes of mutants showing whatever the genetic basis of the effect, alterations in either their cell walls or their behaviour during cell division have also been isolated. Use of these mutants has allowed elucidation of the control of the isoleucine biosynthetic path way.

Temperature sensitive mutants, because they show the defect in metabolism only when grown at the non-permissive temperature, may permit the study of metabolic processes for which constitutive mutants are not obtainable due to their lethal effect on growth. In addition, such mutants are invaluable in the study of cellular biogenesis, and in cases where the thermosensitive product is a protein, may permit discernment of its site of synthesis. In the present context, screening for such temperature sensitive mutants may permit further phenotypic characterisation and categorisation of the mutants isolated.

Temperature sensitive mutants with an altered DNA - RNA polymerase have been isolated in E.coli using a technique strictly analogous to that used

here, with a primary isolation of rifampicin resistant strains followed by a secondary screening amongst these resistant cells for mutants which show temperature sensitivity in their growth pattern in the absence of rifampicin (Reid, 1971). The following chapter lists the results obtained on screening the isolated oligomycin resistant mutants for temperature sensitivity.

Results

The OL^R mutants were screened at 20, 30 and 37°C for alteration of their growth on fermentable and non-fermentable substrates, indicating a temperature sensitive alteration in either general cellular or mitochondrial metabolism. The mutants were also assayed at different temperatures for their resistance to oligomycin. The results presented in Tables 5.1 and 5.2 indicate that none of the class I nor the class II mutants show thermo- or cryo-sensitivity on either YEPG or YEPGly media. At neither 21°C nor 37°C did the growth response of any of the mutants differ from that observed at 30°C - the temperature of mutant isolation.

In contrast to this behaviour, the oligomycin resistance of some of the strains showed marked cryogenicity (Table 5.1), resistance being lost at 21°C. This loss of resistance was only apparent at low temperatures. Incubation of the resistant strains to temperatures above that used in mutant isolation, did not cause any loss of resistance in any of the strains tested. The results obtained when screening was performed at 37°C are shown in Table 5.2.

The cryogenicity of oligomycin resistance, but not growth, is also shown in Plate 1 which represents the results of one of a series of experiments undertaken to demonstrate this phenomenon. One of the characteristics of the cryogenic sensitivity of the oligomycin resistance has been that the clarity with which the effect was demonstrated depends on the concentration of oligomycin in the growth media - the higher the level of oligomycin used, the more decisive is the distinction between the cold sensitive OL^R strains and the cold insensitive OL^R strains. This is clearly demonstrated in both Tables 5.1 and Plate 1. For instance, strains D22B9, D22B15 and D22B16, all of which are resistant to over 10 γ/ml at 30°C fail to show a loss of oligomycin resistance when tested against 2.5 γ/ml oligomycin at 21°C, but show this effect very clearly when tested against 5.0 γ/ml oligomycin at the

TABLE 5.1.

Cryosensitivity of Growth and Resistance at 21°C

The strains to be tested were dropped out onto YEPGly plates containing 0, 2.5 and 5.0 γ/ml oligomycin and also onto YEPG plates. Strains were incubated on all media at both 21°C and 30°C for 3 - 4 days before scoring.

Key

* Poor growth.

TABLE 5.1.

Strain	30°C Oligomycin Resistance to 2.5 γ/ml	30°C Oligomycin Resistance to 5.0 γ/ml	21°C Oligomycin Resistance to 2.5 γ/ml	21°C Oligomycin Resistance to 5.0 γ/ml	30°C Glycerol Growth	21°C Glycerol Growth	Class
D22	-	-	-	-	+	+	
D22Er310	-	-	-	-	+	+	
D6	-	-	-	-	+	+	
D22A1	+	+	+	+	+	+	III
D22A2	+	-	-	-	+	+	I
D22A3	+	+	+	-	+	+	I
D22A4	+	+	-	-	+	+	I
D22A5	+	+	+	+	+	+	I
D22A7	+	+	-	-	+	+	I
D22A9	+	+*	-	-	+	+	I
D22A11	+	+*	+	+*	+	+	I
D22A12	+	+	+	+	+*	+*	I
D22A13	+	+	+	+	+	+	II
D22A14	+	+	+	+	+	+	II
D22A15	+	+	+	+	+	+	II
D22A16	+	+	+	+	+	+	II
D22A18	+	-	+	-	+	+	II
D22A19	+	+	+	+	+	+	II
D22A20	+	+	-	-	+	+	I
D22A21	+	+	+	+	+	+	II
D22A22	+	+	+	+	+	+	II

TABLE 5.1.

Strain	30°C Oligomycin Resistance to 2.5 γ/ml	30°C Oligomycin Resistance to 5.0 γ/ml	21°C Oligomycin Resistance to 2.5 γ/ml	21°C Oligomycin Resistance to 5.0 γ/ml	30°C Glycerol Growth	21°C Glycerol Growth	Class
D22 B1	+	+	+	+	+	+	II
D22 B3	+	+	+	+	+	+	I
D22 B4	+	+	+	(- ?)	+	+	I
D22 B6	+	+	+	-	+	+	I
D22 B8	+	+	+*	-	+	+	I
D22 B9	+	+	+	-	+	+	I
D22 B10	+	+	-	-	+	+	I
D22 B15	+	+	+	-	+	+	I
D22 B16	+	+	+	-	+	+	I
D22 B20	+	+	-	-	+	+	I
D22 B21	+	+	+	(- ?)	+	+	II
D22 B22	+	-	-	-	+	+	I
D22 B23	N.T.	+	N.T.	+	+	+	II
D22 C1	+	+	+	+	+	+	I
D22 C2	+	+	+	+	+	+	II
D22 C4	+	+	+	+	+	+	II

TABLE 5.1.

Strain	30°C Oligomycin Resistance to 2.5 γ/ml	30°C Oligomycin Resistance to 5.0 γ/ml	21°C Oligomycin Resistance to 2.5 γ/ml	21°C Oligomycin Resistance to 5.0 γ/ml	30°C Glycerol Growth	21°C Glycerol Growth	Class
D6 A1	+	+	+	+	+	+	II
D6 A3	+*	-	-	-	+	+	I
D6 A4	+	+	+	-	+	+	I
D6 A5	+	+	+	+	+	+	II
D6 A8	+	+*	-	-	+	+	
D6 A9	+	+	+	-	+	+	I
D6 A10	+	+	+	+	+	+	I
D6 A11	+	-	-	-	+	+	I
D6 A15	+	+*	-	-	+	+*	I
D6 B1	+	+	+	+	+	+	II
D6 B2	+	+	+	+	+	+	II
D6 B3	+	+	+*	+*	+	+*	II
D22 A7 D6<u>11</u>	+	+	+	-	+	+	I
D22 B9 D6<u>C</u>	+	+	+	-	+	+	I

TABLE 5.2.

Thermosensitivity of Growth and Resistance at 37°C

The strains to be tested were dropped out onto YEPGly plates containing 0 and 5.0 γ/ml oligomycin and also onto YEPG plates. Strains were incubated on all the plates at both 30°C and 37°C for 2 - 3 days before scoring.

Key

* Poor growth

TABLE 5.2.

Strain	Growth on Glucose at 30°C	Growth on Glycerol at 30°C	Growth on Glucose at 37°C	Growth on Glycerol at 37°C	Oligomycin Resistance at 30°C	Oligomycin Resistance at 37°C	Class
D22	+	+	+	+	-	-	
D6	+	+	+	+	-	-	
D22 A1	+	+	+	+	+	N.T.	III
D22 A2	+	+	+	+*	+	N.T.	I/a
D22 A3	+	+	+	+	+	+	I/a
D22 A4	+	+	+	+	+	+	I/a
D22 A5	+	+	+	+	+	N.T.	I/b
D22 A7	+	+	+	+	+	+	I/a
D22 A9	+	+	+	+	+	N.T.	I/a
D22 A11	+	+	+	+	+	N.T.	I/b
D22 A12	+	+	+	+	+	N.T.	I/b
D22 A13	+	+	+	+	+	+	II/b
D22 A14	+	+	+	+	+	N.T.	II/b
D22 A15	+	+	+	+	+	+	II/b
D22 A16	+	+	+	+	+	+	II/b
D22 A18	N.T.	N.T.	N.T.	N.T.	N.T.	N.T.	II/b
D22 A19	+	+	+	+	+	+	II/b
D22 A20	+	+	+	+*	+	+	I/a
D22 A21	+	+	+	+	+	+	II/b
D22 A22	+	+	+	+	+	N.T.	II/b

TABLE 5.2.

Strain	Growth on Glucose at 30°C	Growth on Glycerol at 30°C	Growth on Glucose at 37°C	Growth on Glycerol at 37°C	Oligomycin Resistance at 30°C	Oligomycin Resistance at 37°C	Class
D22 B1	+	+	+	+	+	+	II/b
D22 B3	+	+	+	+	+	+	I/b
D22 B4	+	+	+	+	+	+	I/a
D22 B6	+	+	+	+	+	+	I/a
D22 B8	+	+	+	+	+	+	I/a
D22 B9	+	+	+	+	+	+	I/a
D22 B10	+	+	+	+	+	N.T.	I/a
D22 B15	+	+	+	+	+	+	I/a
D22 B16	+	+	+	+	+	+	I/a
D22 B20	+	+	+	+	+	+	I/a
D22 B21	+	+	+	+	+	+	II/a
D22 B22	+	+	+	+	+	N.T.	I/a
D22 B23	+	+	+	+	+	+	II/b
D22 C1	+	+	+	+	+	+	I/b
D22 C2	+	+	+	+	+	+	II/b
D22 C4	+	+	+	+	+	+	II/b

TABLE 5.2.

Strain	Growth on Glucose at 30°C	Growth on Glycerol at 30°C	Growth on Glucose at 37°C	Growth on Glycerol at 37°C	Oligomycin Resistance at 30°C	Oligomycin Resistance at 37°C	Class
D6A1	+	+	+	+	+	N.T.	II/b
D6A3	+	+	+	+	+	+	I/a
D6A4	+	+	+	+	+	N.T.	I/a
D6A5	+	+	+	+	+	N.T.	II/b
D6A8	+	+	+	+	+	N.T.	/a
D6A9	+	+	+	+	+	+	I/a
D6A10	+	+	+	+	+	N.T.	I/b
D6A11	+	+	+	+	+	+	I/a
D6A15	+	+	+	+*	+	N.T.	I/a
D6B1	+	+	+	+	+	+	II/b
D6B2	+	+	+	+	+	+	II/b
D6B3	+	+	+	+	+	+	II/b

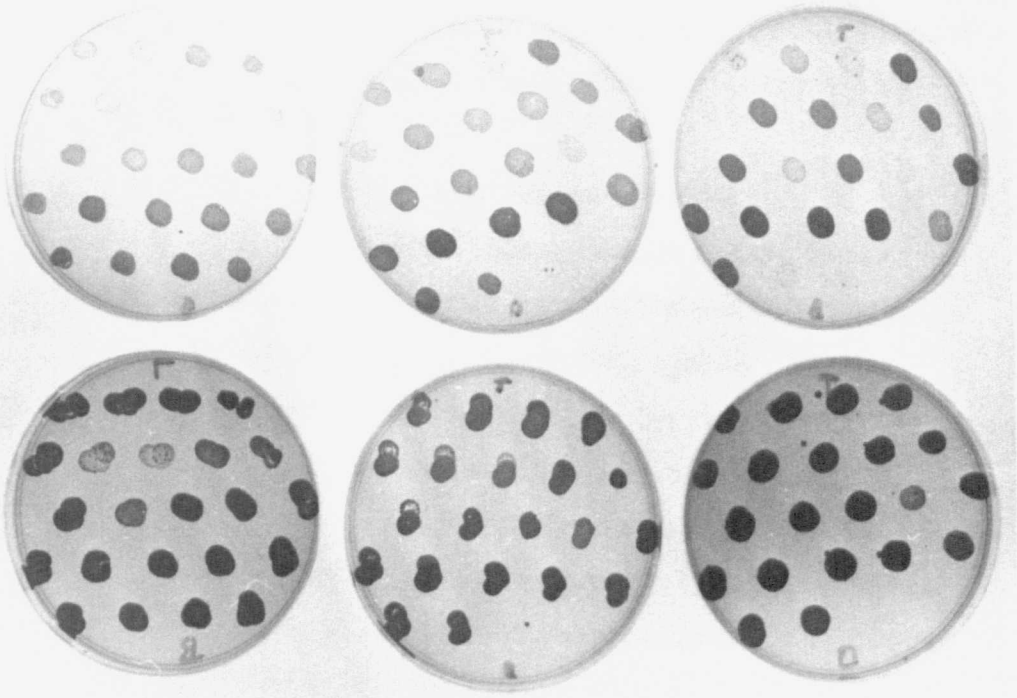


PLATE 1

Assay for Cryogenic Sensitivity of Oligomycin Resistance

L - R Top Row

Glycerol only 21°C	Glycerol + 2.5 γ/ml oligomycin 21°C	Glycerol + 5.0 γ/ml oligomycin 21°C

L - R Bottom Row

Glycerol only 30°C	Glycerol + 2.5 γ/ml oligomycin 30°C	Glycerol + 5.0 γ/ml oligomycin 30°C

same temperature. It may be that in the class I mutants demonstrating this effect, some type of titration effect of oligomycin against the individual cell or against a component involved in the oligomycin resistance of the individual cell is occurring. Some of the genetic results presented in chapter 6 may also be explicable on this basis.

Those mutants whose oligomycin resistance is cold sensitive show, with only one possible exception, similar behaviour with respect to their associated rutamycin resistance, whilst those mutants which do not show this cold sensitivity, similarly fail to show this effect against rutamycin (Tables 5.3 and 5.4). These two tables also analyse the results shown in Table 5.1 with respect to the two classes of oligomycin mutants. It is apparent that the majority of the oligomycin resistant mutants exhibiting cold sensitivity of oligomycin resistance belong to class I, only one strain out of twenty belonging to class II. Within the class I oligomycin resistant mutants, approximately two-thirds of the mutants show such cold sensitivity. It appears therefore, that the mutation(s) that has occurred in this class tends to predispose the resulting oligomycin resistance to phenotypic loss on exposure to low temperatures.

In view of the observation that the loss of oligomycin resistance on exposure to low temperatures was to some extent dependent on the level of oligomycin in the test medium, experiments were undertaken to test those class I and II OL^R mutants which were not apparently cold sensitive against increased levels of oligomycin and rutamycin. Those strains of both classes which had not lost their resistance on growth at 21°C in the previous assays carried out with 5.0 γ/ml oligomycin, were fully resistant at 21°C when the assay was carried out with 10.0 γ/ml oligomycin and up to 100.0 γ/ml of rutamycin. It must therefore be concluded that these strains are truly resistant at this temperature - especially as none of the cold sensitive mutants has shown resistance to greater than 10.0 γ/ml of rutamycin at 21°C (Table 5.3). The cold sensitive class I mutants did not lose their associated cross resistance to mikamycin, chloramphenicol, TTFB and triethyltin on testing at 21°C (Table 5.5 ; Plate 2). Results (not shown) obtained using the class II mutants as well as the parental strains D6 and

TABLE 5.3.

Effect of Temperature on the Oligomycin and Rutamycin Resistances of the Class I Mutants

Strains were tested for their oligomycin and rutamycin resistance at 21°C and 30°C by dropping out onto YEPGly plates containing 0, 2.5, 5.0 and 10.0 γ/ml oligomycin and 0, 5.0, 10.0, 25.0, 50.0 and 100.0 γ/ml rutamycin. Plates were scored after 4 days incubation at either 21°C or 30°C.

Ratio of temperature conditional : non-conditional resistant Class I strains = 19:8

TABLE 5.3.

Strain	Class	Oligomycin Resistance Temperature Sensitivity	Rutamycin Resistance at 30°C	Rutamycin Resistance at 21°C
D22 A1	III	None	N.T.	N.T.
D22 A2	I	Sensitive	N.T.	N.T.
D22 A3	I	Sensitive	50.0	10.0
D22 A4	I	Sensitive	25.0	10.0
D22 A5	I	None	100.0	100.0
D22 A7	I	Sensitive	100.0	10.0
D22 A9	I	Sensitive	N.T.	N.T.
D22 A11	I	None	N.T.	N.T.
D22 A12	I	None	50 - 100.0	100.0
D22 A20	I	Sensitive	25 - 50.0	10.0
D22 B3	I	None	> 50.0	> 50.0
D22 B4	I	Sensitive	5 - 10.0	2.5
D22 B6	I	Sensitive	100.0	10.0
D22 B8	I	Sensitive	25.0	10.0
D22 B9	I	Sensitive	25 - 50.0	10.0
D22 B10	I	Sensitive	N.T.	N.T.
D22 B15	I	Sensitive	100.0	10.0
D22 B16	I	Sensitive	50.0	10.0
D22 B20	I	Sensitive	5.0	5.0
D22 B22	I	Sensitive	N.T.	N.T.
D22 C1	I	None	> 50.0	> 50.0
D6 A3	I	Sensitive	N.T.	N.T.
D6 A4	I	Sensitive	N.T.	N.T.
D6 A9	I	Sensitive	N.T.	N.T.
D6 A10	I	None	N.T.	N.T.
D6 A11	I	Sensitive	N.T.	N.T.
D6 A15	I	Sensitive	N.T.	N.T.
D22 B8 D6 12	I	Sensitive	10.0	2.5

TABLE 5.4.

Effect of Temperature on the Oligomycin and Rutamycin Resistances of the Class II Mutants

Strains were tested for their oligomycin and rutamycin resistance at 21°C and 30°C by dropping out onto YEPGly plates containing 0, 2.5, 5.0 and 10.0 γ/ml oligomycin and 0, 5.0, 10.0, 25.0, 50.0 and 100.0 γ/ml rutamycin. Plates were scored after 4 days incubation at either 21°C or 30°C.

Ratio of temperature conditional : non-conditional resistant Class II strains = 1 : 17

TABLE 5.4.

Strain	Class	Oligomycin Resistance Temperature Sensitivity	Rutamycin Resistance at 30°C γ/ml	Rutamycin Resistance at 21°C γ/ml
D22 A13	II	None	25.0 - 50.0	25.0 - 50.0
D22 A14	II	None	N.T.	N.T.
D22 A15	II	None	25.0 - 50.0	25.0 - 50.0
D22 A16	II	None	> 100.0	> 100.0
D22 A18	II	None	N.T.	N.T.
D22 A19	II	None	> 100.0	> 100.0
D22 A21	II	None	N.T.	N.T.
D22 A22	II	None	N.T.	N.T.
D22 B1	II	None	> 100.0	> 100.0
D22 B21	II	Sensitive	> 100.0	> 100.0 (?)
D22 B23	II	None	N.T.	N.T.
D22 C2	II	None	> 100.0	> 100.0
D22 C4	II	None	50.0 - 100.0	50.0 - 100.0
D6 A1	II	None	N.T.	N.T.
D6 A5	II	None	N.T.	N.T.
D6 B1	II	None	N.T.	N.T.
D6 B2	II	None	N.T.	N.T.
D6 B3	II	(None ?)	N.T.	N.T.

TABLE 5.5.

Cryogenicity of Associated Cross Resistances of the Class I and Class III Resistant Mutants

The Class I and III mutants were tested for the temperature sensitivity of their resistance to oligomycin, chloramphenicol and mikamycin by dropping out all suspensions onto YEPGly media ± various drugs. The drug concentrations used were :

Oligomycin, 0, 2.5 and 5.0 γ/ml

Chloramphenicol, 0, 1.0 and 2.0 mg/ml

Mikamycin, 0, 0.005 and 0.01 mg/ml

Triethyltin, 0, 5.0 and 10.0 γ/ml

TTFB, 0, 10.0, 20.0 and 30.0 γ/ml

Key

* Growth poor at 21°C on YEPGly

TABLE 5.5.

Cryogenicity of Associated Cross Resistances of the Class I and Class III Resistant Mutants

Strain	Cryogenicity of Oligomycin Resistance	Mikamycin Resistance mg/ml		Chloramphenicol Resistance mg/ml		Triethyltin Resistance γ/ml		TTFB Resistance γ/ml	
		30°C	21°C	30°C	21°C	30°C	21°C	30°C	21°C
D22		< 0.005	< 0.005	< 1.0	< 1.0	0.5	N.T.	8.25	N.T.
D6		< 0.005	< 0.005	0.5 - 1.0	0.5 - 1.0	0.5	N.T.	2.5	N.T.
D22 A1	-	0.01	0.01	< 1.0	< 1.0	< 5.0	< 5.0	10 - 20.0	> 10.0
D22 A2	+	0.005	0.005	2.0	2.0	> 10.0	> 10.0	> 30.0	> 10.0
D22 A3	+	0.01	0.01	2.0	2.0	5 - 10.0	5 - 10.0	20.0	> 10.0
D22 A4	+	0.01	0.01	2.0	2.0	5.0	5 - 10.0	20.0	> 10.0
D22 A7	+	N.T.	N.T.	N.T.	N.T.	5 - 10.0	> 10.0	> 30.0	> 10.0
D22 A7 D6 11	+	0.01	0.01	2.0	2.0	N.T.	N.T.	N.T.	N.T.
D22 A9	+	N.T.	N.T.	N.T.	N.T.	5.0	5 - 10.0	20.0	> 10.0
D22 A12	-	0.01	0.01	2.0	2.0	5 - 10.0	5 - 10.0	20 - 30.0	> 10.0

TABLE 5.5.

Strain	Cryogenicity of Oligomycin Resistance	Mikamycin Resistance mg/ml		Chloramphenicol Resistance mg/ml		Triethyltin Resistance γ/ml		TTFB Resistance γ/ml	
		30°C	21°C	30°C	21°C	30°C	21°C	30°C	21°C
D22 B3	-	0.01	0.01	2.0	2.0	5 - 10.0	> 10.0	> 30.0	> 10.0
D22 B4	-	0.01	0.01	2.0	2.0	N.T.	N.T.	N.T.	N.T.
D22 B6	+	0.01	0.01	2.0	2.0	5 - 10.0	> 10.0	20.0	> 10.0
D22 B8	+	0.01	0.01	2.0	2.0	5.0	5 - 10.0	10.0	> 10.0
D22 B9	+	0.01	0.01	2.0	2.0	5 - 10.0	5 - 10.0	20 - 30.0	> 10.0
D22 B9D6 C	+	0.01	0.01	2.0	2.0	N.T.	N.T.	N.T.	N.T.
D22 B10	+	0.01	0.01	2.0	2.0	N.T.	N.T.	N.T.	N.T.
D22 B15	+	0.01	0.01	2.0	2.0	5 - 10.0	> 10.0	> 30.0	> 10.0
D22 B16	+	0.01	0.01	2.0	2.0	5.0	> 10.0	20.0	> 10.0
D22 C1	-	0.01	0.01	2.0	2.0	> 10.0	> 10.0	20 - 30.0	> 10.0
D6 A4	+	0.01	0.01	2.0	2.0	< 5.0	5.0	< 10.0	> 10.0
D6 A9	+	0.01	0.01	2.0	2.0	< 5.0	5.0	< 10.0	> 10.0
D6 A15	+	0.01	0.01 - 0.005	2.0	2.0 - 1.0	N.T.	N.T.	N.T.	N.T.

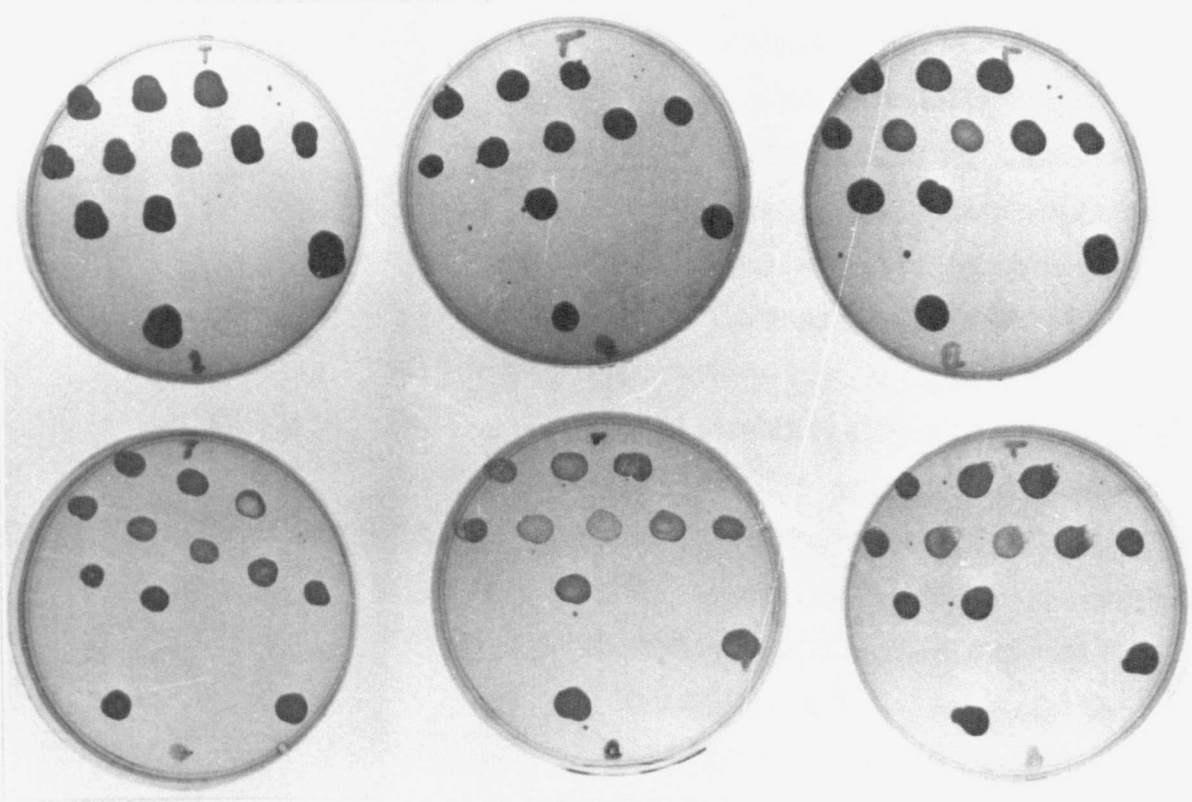


PLATE 2

Assay for Cryogenic Sensitivity of Class I Cross Resistances

L - R Top Row

30°C	Mikamycin 0.01 mg/ml	Chloramphenicol 2.0 mg/ml	Mikamycin 0.005 mg/ml

L - R Bottom Row

21°C	Mikamycin 0.01 mg/ml	Chloramphenicol 2.0 mg/ml	Mikamycin 0.005 mg/ml

D22 indicate that there was no significant change in the levels of chloramphenicol and mikamycin necessary to inhibit non-fermentable growth at 21°C as opposed to 30°C. The failure to find any loss of cross resistance in the class I mutants to these inhibitors is not therefore due to a diminution in the inhibitory activity of a given amount of these compounds at the lower temperatures. Observations made by Mr. W. Lancashire (private communication) and similar observations made during these experiments, do indicate, however, that chloro CCP, triethyl-tin and TTFB become less active inhibitors as the temperature is lowered, so the results observed with these compounds could be due, in part, to this effect.

The cold sensitivity of some resistant strains to oligomycin and rutamycin cannot be due to an effect such as described for TTFB, etc. as otherwise at each of the different temperatures all the mutants would be expected to behave similarly. Analysis of the results of the oligomycin and rutamycin resistance levels of both non-cold sensitive OL^R strains such as D22 A18 and D22 A13 (Table 5.4) as well as the OL^S parental strains D6 and D22, have shown that there is no shift in the level of oligomycin required to inhibit glycerol supported growth when the temperature is changed from 30°C to 21°C.

On the basis of these results, it appears possible to divide the two classes of mutants already identified into two further phenotypic sub-classes. These have been called 'a' and 'b'. Suffix 'a' is applied to mutants of either class whose resistance is cold sensitive and suffix 'b' to mutants whose resistance is not cold labile.

Experiments have been undertaken to demonstrate this loss of oligomycin resistance on exposure to temperatures around 20°C in liquid medium, as a preliminary to carrying out biochemical studies relating to both the nature of the gene product(s) involved in conferring resistance and whether this product or the enzyme involved in its synthesis is synthesised on the mitoribosomes or the cytoribosomes. Figure 5.1 shows the results of an experiment carried out with strain D22 B9 (OL^R 1a) which was grown at 20°C on YEPGly medium in the presence and absence of oligomycin. At 20°C growth proceeds after the lag period quite normally in the absence of oligomycin. In its presence, however, growth is totally inhibited. On alteration of the temperature to 30°C, growth resumes in the oligomycin treated culture some 2 - 4 hours after the 20 - 30°C

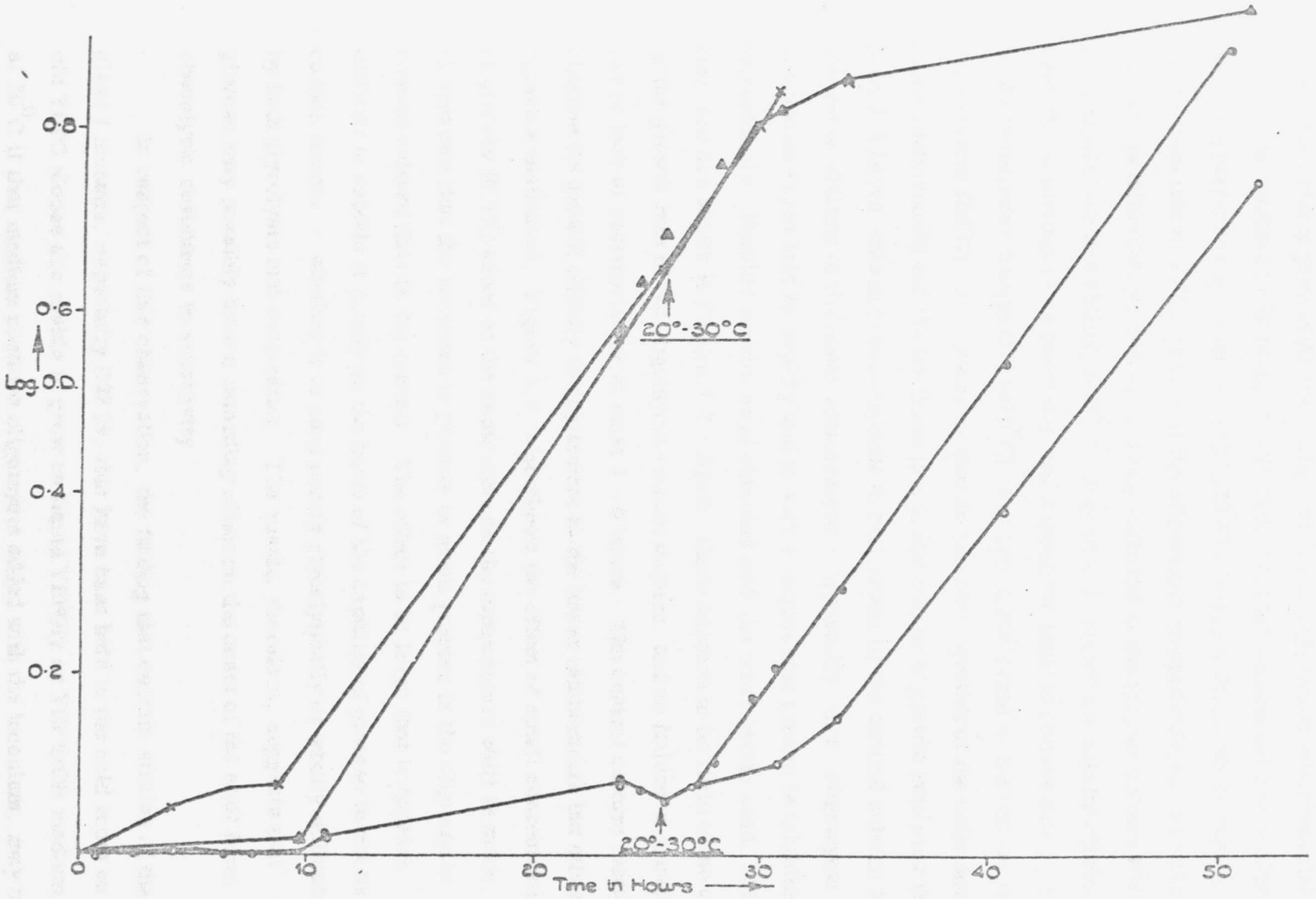
LEGEND TO FIGURE 5.1.

Temperature Sensitivity of Oligomycin Resistance of D22B9 -(20/30°).

A 0.5% inoculum of D22B9 (OL^R Class Ia) taken from a stationary phase YEPG culture was added to 25 ml of YEPGly media ± 5.0 γ/ml oligomycin contained in 500 ml sidearm flasks and the flasks grown at 20°C on an orbital shaker. After 26 hours the flasks were transferred to a 30°C shaker and one of each pair of flasks (± oligomycin) simultaneously had 0.1% glucose added to them. The growth of the cultures was followed using an EEL colorimeter fitted with a 607 filter.

Key

- ○ + oligomycin
- × - oligomycin
- ● + oligomycin + 0.1% glucose added on temperature shift
- ▲ - oligomycin + 0.1% glucose added on temperature shift.


0·8
0·6
0·4
0·2
Log O.D.
20°-30°C
20°-30°C
0
10
20
30
40
50
Time in Hours

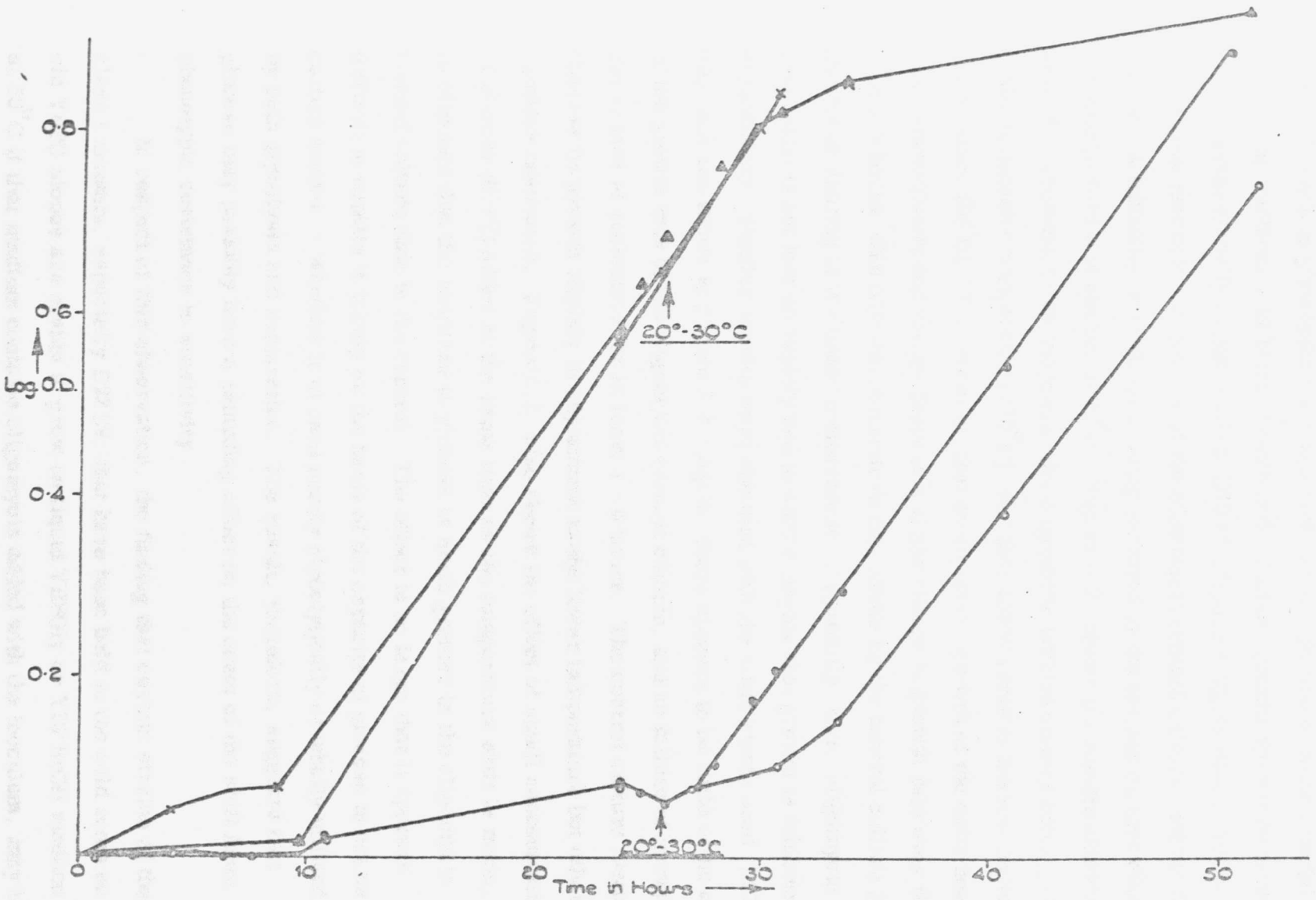
LEGEND TO FIGURE 5.1.

Temperature Sensitivity of Oligomycin Resistance of D22B9 -(20/30°).

A 0.5% inoculum of D22B9 (OL^R Class Ia) taken from a stationary phase YEPG culture was added to 25 ml of YEPGly media ± 5.0 γ/ml oligomycin contained in 500 ml sidearm flasks and the flasks grown at 20°C on an orbital shaker. After 26 hours the flasks were transferred to a 30°C shaker and one of each pair of flasks (± oligomycin) simultaneously had 0.1% glucose added to them. The growth of the cultures was followed using an EEL colorimeter fitted with a 607 filter.

Key

- ○ + oligomycin
- × - oligomycin
- • + oligomycin + 0.1% glucose added on temperature shift
- ▲ - oligomycin + 0.1% glucose added on temperature shift.


0·8
0·6
0·4
0·2
Log O.D.
20°-30°C
20°-30°C
0
10
20
30
40
50
Time in Hours

temperature transfer. If small amounts of glucose are added at the same time as the temperature shift is performed, the growth resumes within 2 hours, presumably due to glycolysis providing cellular energy whilst mitochondrial oligomycin resistance is being established. Similar results have been obtained with the other class Ia mutant tested, D22 A7 (Figure 5.2), in which, again, it can be seen that at 20°C, growth of the oligomycin treated culture, but not the control, is inhibited, growth again being restored to the former culture when the temperature was shifted to 30°C. Figure 5.2. shows the results obtained when the experiment was reversed the oligomycin treated culture being grown at the permissive temperature (30°C) and then transferred to the non-permissive temperature (20°C). It is apparent that on transfer, growth of the cells does not cease immediately and though there is a slight change in growth rate over the first 2.5 hours, this only corresponds to that shown by the control culture (not shown) on shifting to the lower temperature. Apparently, then, oligomycin resistance is not lost so rapidly and to such a degree that growth is inhibited immediately. Similar results were obtained with the other strain used, D22 B9 (Ia), and are shown in Figure 5.3. Again, there appears to be little or no change in the growth rate of the oligomycin treated culture, and no failure of growth due to loss of resistance for at least 4 - 6 hours. The control culture meanwhile changes its growth slightly in adjustment to the lower temperature but otherwise remains unaffected. Figure 5.3. also shows the effect of small concentrations of glucose (0.1%) added at the same time as the temperature shift is made. It is apparent that the response to glucose is much greater in the oligomycin treated culture than in the control. The effect is so large that it appears difficult to explain it purely on the basis of the capacity of glucose to act as a carbon source - whether it is used purely glycolytically or wholly utilised by both glycolysis and respiration. The result, therefore, suggests that glucose may possibly have a retarding effect on the onset of the shift from phenotypic resistance to sensitivity.

In respect of this observation, the finding that certain strains of these class I mutants, especially D22 B9, that have been held in the cold room on old YEPG slopes are unable to grow on liquid YEPGly or YEP EtOH medium at 30°C if that medium contains oligomycin added with the inoculum, may be

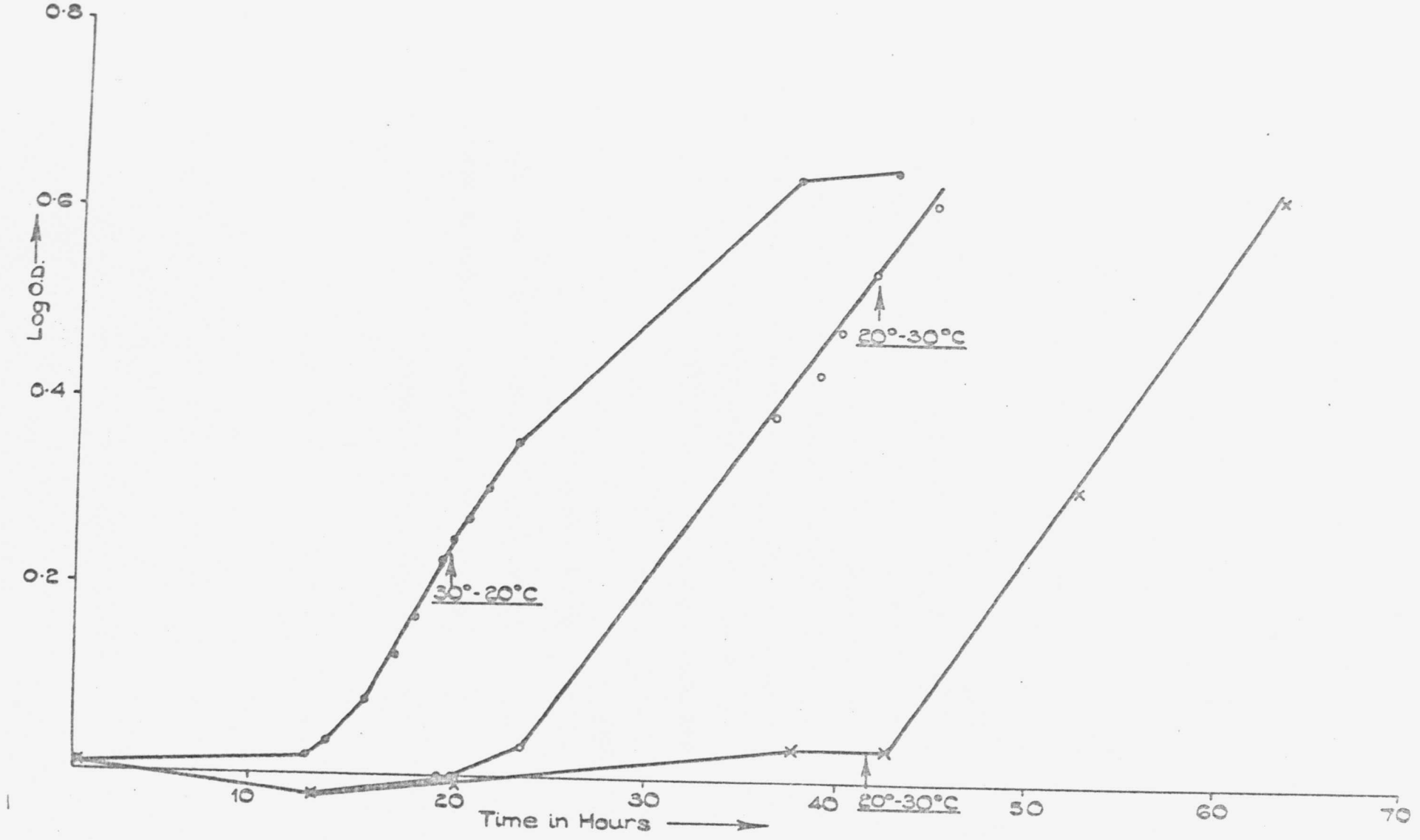
LEGEND TO FIGURE 5.2.

Temperature Sensitivity of Oligomycin Resistance of D22A7 -(30/20°).

A standard 1% inoculum of strain D22 A7 (OL^R Class Ia) was inoculated into 50 ml of YEPGly medium ± 5.0 γ/ml oligomycin contained in 500 ml sidearm flasks and the flasks grown at 20°C or 30°C on an orbital shaker. The flasks were transferred from 30° - 20°C or vice versa at the times indicated on the graph. The rate of growth was followed using an EEL colorimeter fitted with a 607 filter.

Key

- • + oligomycin 30°C - 20°C
- ○ - oligomycin 20°C - 30°C
- × + oligomycin 20°C - 30°C


0·8
0·6
0·4
0·2
Log o.d.
30°-20°C
20°-30°C
20°-30°C
10
20
30
40
50
60
70
Time in Hours

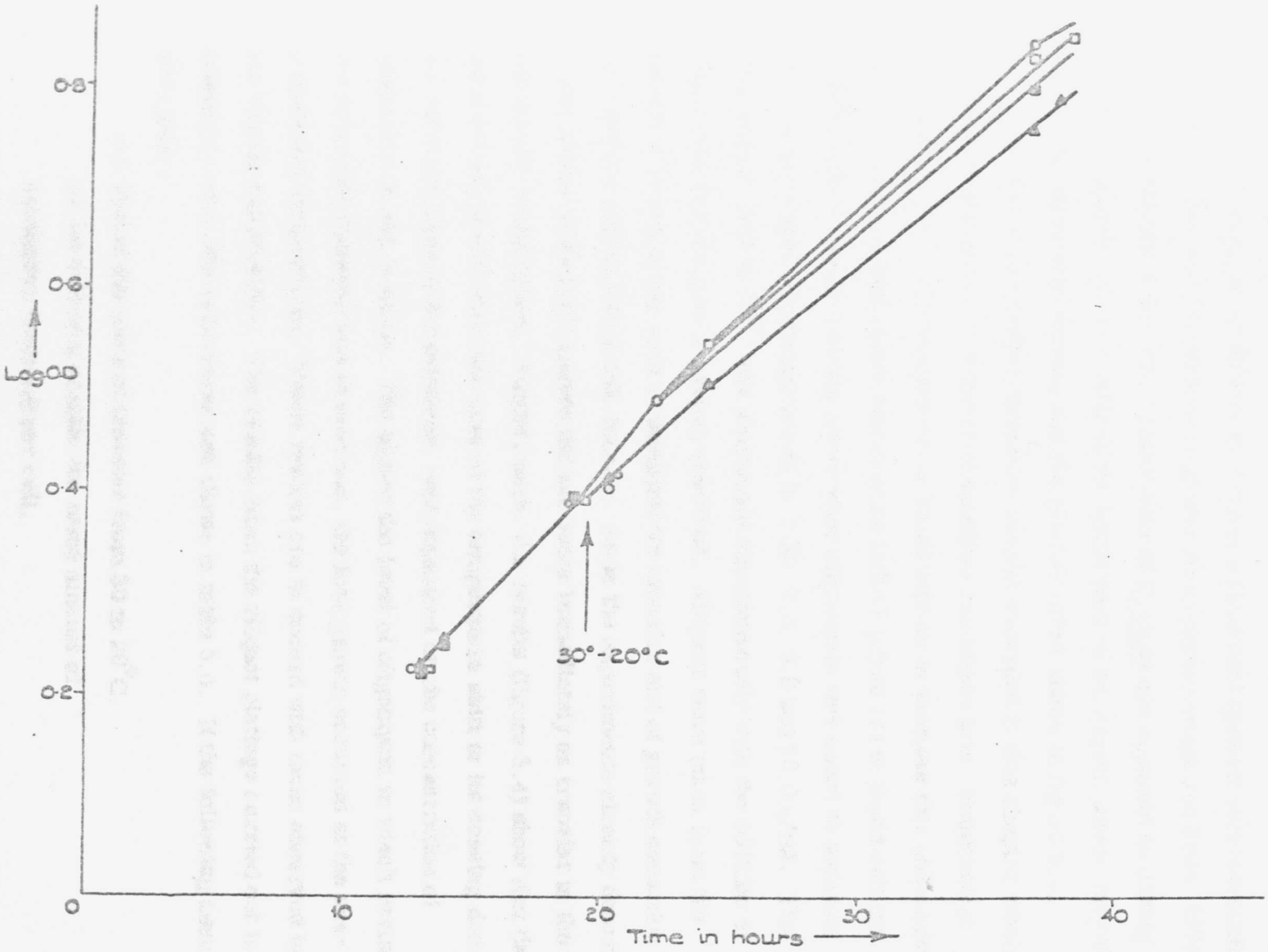
LEGEND TO FIGURE 5.3.

Temperature Sensitivity of Oligomycin Resistance of D22B9 - (30/20°).

A standard 1% inoculum of strain D22 B9 (Class Ia) was inoculated into 50 ml of YEPGly medium contained in 500 ml sidearm flasks ± 5.0 γ/ml oligomycin and the cultures grown at 30°C on an orbital shaker. After 18 hours the flasks were transferred to a 20°C shaker and one of each pair of flasks (± oligomycin) simultaneously had 0.1% glucose (final concentration) added to it. The rate of growth was followed using an EEL colorimeter with a 607 filter.

Key

- ■ D22 B9 - oligomycin
- ○ D22 B9 - oligomycin + 0.1% glucose (on shift)
- □ D22 B9 + 5.0 γ/ml oligomycin + 0.1% glucose (on shift)
- ▲ D22 B9 + 5.0 γ/ml oligomycin


0·8
0·6
0·4
0·2
0
Log OD
10
20
30
40
Time in hours
30°-20°C

significant, especially as this effect has not been noticed if the YEPG stock slopes are fresh, or if the oligomycin is added after the inoculum, growth at 30°C occurring prior to its addition. Similar observations have been recorded in respect of some yeast strains by Coen and Slonimski (private communication). The observation made by McMurrough and Rose (McMurrough and Rose, 1971) that large changes in the lipid composition of C.utilis are apparent on adding glucose to starved cells or cells in the latter part of the growth phase, may be relevant to these observations and the glucose effect shown in figure 5.3.

In view of the earlier screening results recorded in this chapter relating to the quantitative nature of the cold sensitive resistance loss, temperature shift experiments were carried out in liquid culture to examine this phenomenon further. The approach taken was to grow D22 A7 (class I a) in liquid culture at 30°C until it was in mid-log phase when oligomycin was added to separate flasks to give final concentrations of 0, 1.25, 2.5, 5.0 and 10.0 γ/ml. The temperature shift to 20°C was performed simultaneously with the addition of oligomycin and the growth pattern observed. Aliquots were taken from the individual flasks at the point of temperature transfer and of growth cessation and used for estimation of cell density. As in the experiments already described, it was observed that cell growth did not cease immediately on transfer to the non-permissive temperature. Furthermore, the results (figure 5.4) show that the extent of the growth from the time of the temperature shift to its ceasing due to the onset of oligomycin sensitivity, was dependent on the concentration of oligomycin in the medium. The higher the level of oligomycin to which resistance was required if growth was to continue, the less growth occurred at the non-permissive temperature. These results are in accord with those observed in the plating experiments. The results from the aliquot platings carried out in connection with this experiment are shown in table 5.6. If the following assumptions are made :

(1) that at the point of transfer from 30 to 20°C, all the cultures contain the same amount of resistance factor(s) per cell,

(2) that there is negligible turnover of this factor(s) in the absence of growth,

Fig.5.4Temperature Sensitivity of Oligomycin Resistance - Oligomycin Dosage Effects.

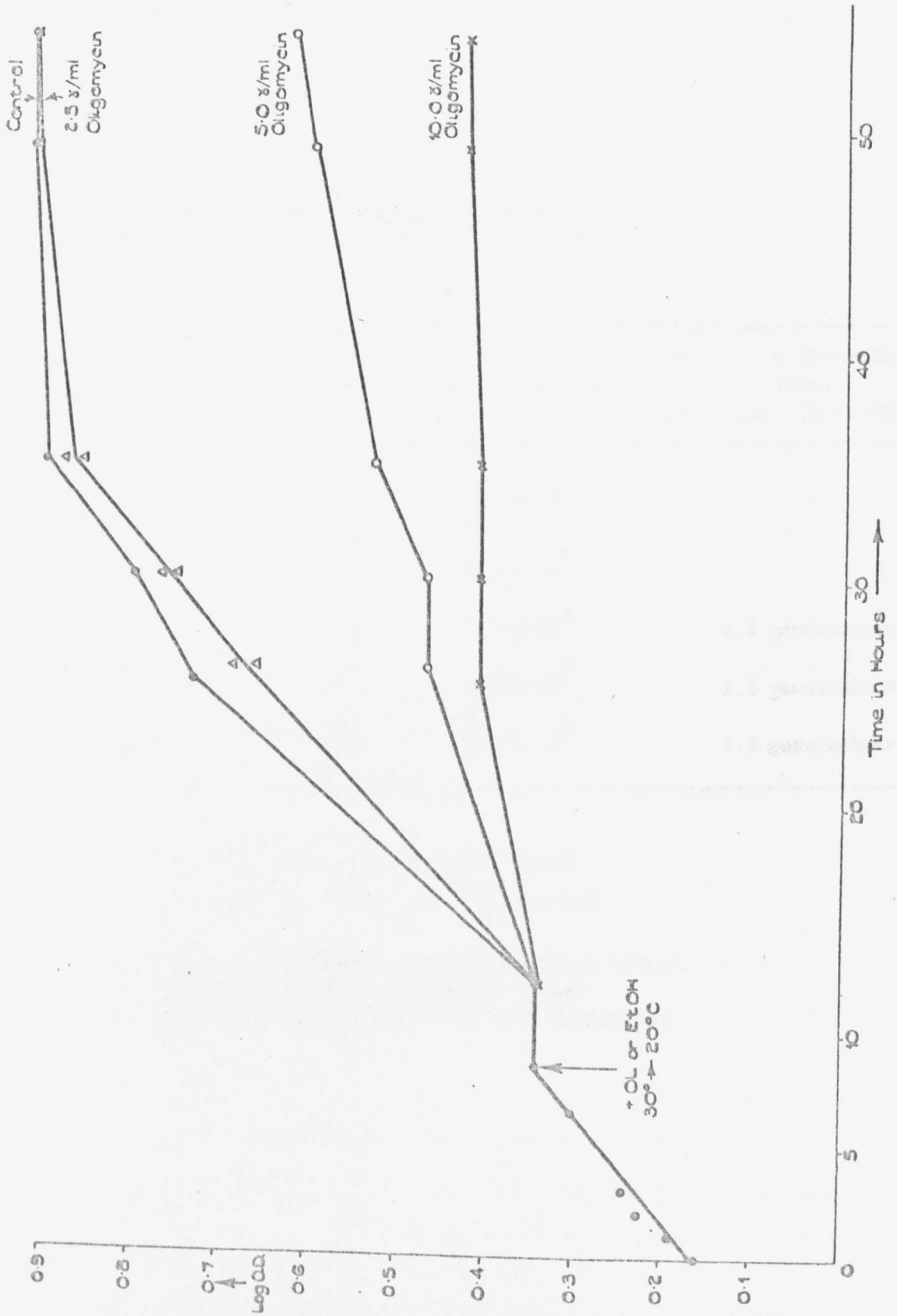


TABLE 5.6.

Concentration of 'Resistance Factor'

Sampling Time	Oligomycin Concentration mg/ml	No. of Cells/ml	No. of Cell Generations from Transition → End of Growth
Point of $30^{\circ} \rightarrow 20^{\circ}C$ transition	-	1.3×10^{7}	-
End of Growth	-	7.0×10^{8}	-
End of Growth	1.25	2.6×10^{8}	4.2 generations
End of Growth	5.0	8.0×10^{7}	2.5 generations
End of Growth	10.0	3.9×10^{7}	1.5 generations

Concentration of 'factor' (calculated)
$= 5.0 \times 10^{-12} - 2.0 \times 10^{-13}$ moles/cell

Concentration of cytochrome c (calculated from data of Sherman, Taber and Campbell quoted Sherman and Stewart, 1971) $= 5.0 \times 10^{-14}$ moles/cell

(3) that no further active 'resistance' factor(s) are made after transfer to 20°C,

then a dilution model may be applied to the data, in order to try to calculate the amount of resistance factor present at 30°C. The calculations are shown in Table 5.6.

As a control to these results obtained with the class Ia mutants, D22 B9 and D22 A7, class Ib and IIb mutants were shown not to possess cold sensitive oligomycin resistance in liquid medium. Figure 5.5. for instance, shows the result of such experiments using strain D22 A12 (class Ib) in temperature shift experiments from 30°C to 20°C and vice versa. No indication of any cold sensitivity of resistance was found.

Discussion

Unlike the majority of purified enzymes, the ATPase is known to be cold labile (Racker, 1965) and, in addition, to become cold labile only during the purification process. This suggests that interaction of the ATPase complex with other membrane components is capable of stabilising or protecting the enzyme in its native state from the effects of low temperature. The finding that the majority of mutants of class I are cold rather than heat sensitive for their oligomycin resistance suggests a possible analogy between these two observations. It must, however, be remembered that there is no evidence at the moment from the class Ia mutants to suggest that the ATPase level in these mutants is lowered after growth at 20°C.

The cold sensitivity of the class Ia mutants appears to be a quantitative rather than a qualitative effect (Figure 5.4) but this has yet to be confirmed in the case of the class IIa mutant. The observations made in regard to the class Ia mutants (Figure 5.4.) strongly resemble, in some ways, the results obtained in crossing the class I mutants by oligomycin sensitive strains when the amount of growth resistance to oligomycin of the diploids was strictly dependent on the levels of oligomycin in the growth media (chapter 6). The finding of cold sensitive yeast mutants has been reported by Weislogel and Butow (Weislogel and Butow, 1970) and also by Hartwell et al. (Hartwell, et al., 1970) who found at least some of their mutants selected for temperature conditional at 36°C were also conditional at 13°C. These

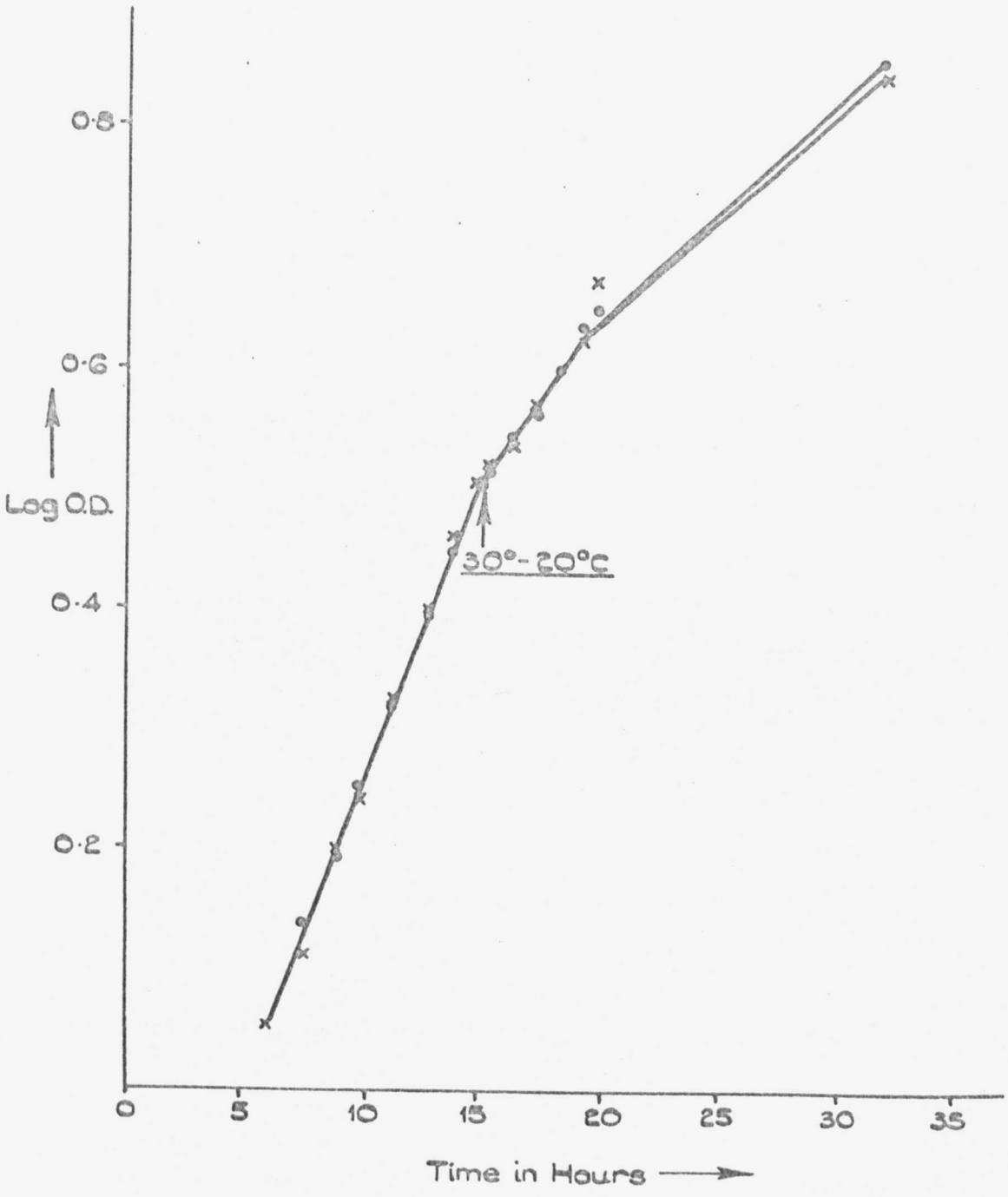
LEGEND TO FIGURE 5.5.

Effect of Temperature on Oligomycin Resistance of D22A12.

A standard inoculum of D22 A12 (Class Ib, YEPG grown) was inoculated into 100 ml of YEPGly contained in 500 ml sidearm flasks $\pm\gamma$/ml oligomycin and the cultures grown at 30°C on an orbital shaker. When the flasks were in mid-log phase, they were transferred to a 20°C shaker and growth allowed to continue. Growth rate was monitored using an EEL colorimeter fitted with a 607 filter. The final concentration of ethanol in the flasks was 1%.

Key

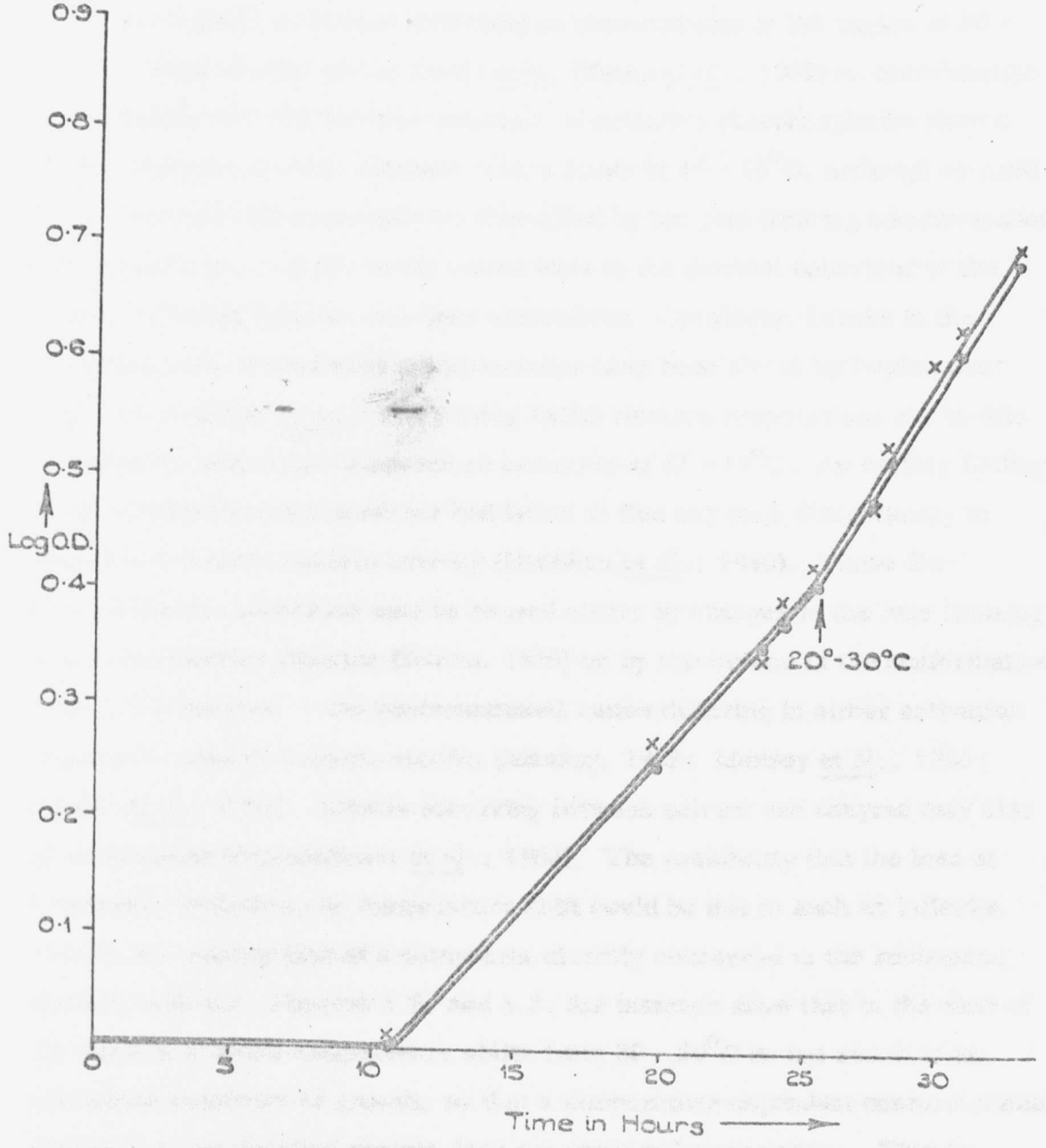
- • + 5 γ/ml oligomycin
- × - 5 γ/ml oligomycin


0·8
0·6
0·4
0·2
Log O.D.
30°-20°C
0
5
10
15
20
25
30
35
Time in Hours

LEGEND FOR FIGURE 5.6.

Effect of Temperature on Oligomycin Resistance of D22A12.

As for Figure 5.5. but transfer from $20^{\circ} \rightarrow 30^{\circ}C$


0·9
0·8
0·7
0·6
0·5
0·4
0·3
0·2
0·1
Log O.D.
0
5
10
15
20
25
30
Time in Hours
20°-30°C

mutants were apparently altered in their ribosome function and similar cold sensitive ribosomal mutants of E.coli have been reported (Guthrie et al., 1969). Schuurman Steckhoven et al. (Schuurman Steckhoven et al., 1971) have described data in which it appears that, in vitro, the majority of energy driven reversal reactions in sub-mitochondrial particles have distinct breaks in their Arrhenius plots, the majority of breaks occurring at temperatures in the region of 30 - 34°C. Studies carried out by Kemp et al. (Kemp et al., 1969) on mitochondria have indicated that the forward reactions of oxidative phosphorylation show a similar biphasic thermal response with a break at 16 - 18°C, although as most of the reactions will apparently be controlled by the rate limiting adenine nucleotide translocase, it is not really known what is the thermal behaviour of the oxidative phosphorylation reactions themselves. Similarly, breaks in the Arrhenius polts of succinate dehydrogenase have been shown by Zeylemaker et al. (Zeylemaker et al., 1971) using Keilin Hartree preparations and in this case also the transition temperature occurred at 17 - 19°C. An earlier finding on heart mitochondrial particles had failed to find any such discontinuity in regard to succinate oxidase activity (Hadidian et al., 1940). These discontinuities or transitions may be caused either by changes in the rate limiting step of the enzyme reaction (Stearn, 1959) or by transitions in the conformational state of the enzyme - the conformational states differing in either activation enthalpy or catalytic centre activity (Massey, 1953; Massey et al., 1966; Koster et al., 1968). Effects occurring between solvent and enzyme may also be responsible (Oppenheimer et al., 1960). The possibility that the loss of oligomycin resistance on temperature shift could be due to such an inflexion point in the activity plot of a component directly concerned in the resistance appears unlikely. Figures 5.2. and 5.3. for instance show that in the case of the class Ia mutants temperature shifts from 30 - 20°C do not result in an immediate cessation of growth, so that a temperature dependent conformational change in a pre-existing protein does not seem to be occurring. This is supported by the findings of J. R. Turner (private communication) who has extracted mitochondria from both the wild type strain D22 and a class Ia mutant grown at 30°C and conducted ATPase assays with these two mitochondrial preparations at both 30° and 20°C. Although the resistance to oligomycin of the

ATPase of both preparations decreased on incubation at 20°C, no differentiation in the behaviour of the class Ia from that of the wild type strain was apparent. It cannot be ruled out, however, on the basis of either of these types of data that the effect is one of altering the conformation/activity of the 'resistant' components of the mitochondria, but that this temperature sensitive switch can only occur before it is incorporated into an organelle - on incorporation it being constrained from any such changes by the physical restraints of being part of a multi-component assembly. Similarly, the temperature sensitivity could be due to an impaired integration of the 'changed factor' into the mitochondrial assembly at the lower temperature either through a change in the factor's conformation itself or that of the rest of the mitochondrial membrane. So long as the binding was essentially thermostable once made, then this model can also not be ruled out by our data. In respect of this, changes in mitochondrial, and probably mitochondrial membrane conformation, dependent on the energy state of the surrounding milieu, are well known (Hackenbroeck, 1968; Stoner and Sirak, 1969; Weber and Blair, 1969). The data in Figures 5.2. and 5.3. may alternatively be explained on the basis that the resistance factor is synthesised either in the resistant or non resistant conformations, depending on the temperature, and is unable to alter its conformations after synthesis is completed. In this case experiments in progress with chloramphenicol and cycloheximide may prove invaluable in studying the resistance factor(s) and its site of synthesis. A further possibility is that the thermosensitive product is not directly related to the resistance but synthesises or regulates the synthesis of the ultimate resistance factor. This, as already suggested could be a lipid membrane component. In this respect it is known that large changes in the composition of the cell fatty acids and lipids do occur, depending on the conditions under which the cells were grown. The number of experimentally alterable conditions which have been shown to influence the lipid composition of the yeast cell include temperature, growth rate, aeration, composition of the growth medium and the age of the cells (Farrell and Rose, 1967; Kates, 1964; Kates, 1966; Marr and Ingraham, 1962; Babij et al., 1969, Brown and Rose, 1969; Jollow et al., 1968; McMurrough and Rose, 1971). Apart from the reports of

absolute changes in fatty acid and lipid composition due to changes in the growing temperature (Farrell and Rose, 1967 ; McMurrough and Rose, 1971 ; Kates, 1964). Melchior et al (Melchior et al., 1970) and Rottem et al (Rottem et al., 1970) have shown that these changes may affect membrane behaviour quite drastically. These workers have found that changes in the fatty acid composition of the membrane caused by lowering the growth temperature, appear to decrease the 'fluidity' of the cell membrane as measured by both thermal transition experiments and the use of E.P.R. on spin labelled fatty acids incorporated into the membranes.

The data presented in this chapter showing that glycerol supported growth is unaffected at 21°C, only the oligomycin resistance being lost, rules out the possibility that these mutants are similar to those described by Weislogel and Butow (Weislogel and Butow, 1970) who have isolated a mutant which at low temperature, buds off such large numbers of petites that growth on non-fermentable substrates ceases. As oligomycin does not increase the petite frequency of the oligomycin resistant strains at 30°C (Chapter 7), the possibility that at 21°C it is having this effect leading to a lack of growth on oligomycin YEPGly medium, seems unlikely even though it cannot be formally ruled out on the evidence to date.

A further thermosensitive yeast mutant of interest which is phenotypically petite at the non-permissive temperature has been reported by Puglisi et al (Puglisi et al., 1970). This mutant, however, which shows nuclear inheritance, is thermosensitive in regard to its coenzyme Q biosynthesis and the 'petite' phenotype involving the loss of cytochromes $a + a_3$ and b is a secondary effect of this primary thermosensitive lesion. The results shown in Table 5.1. demonstrate one other important fact in addition to those already discussed and that is, that in the two cases recorded. D22 A7 D6 11 and D22 B9 D6 C, where class Ia mutants have been crossed with a sensitive OL^S tester and haploids recovered from this diploid by sporulation, not only is the cross resistance characteristic transmitted along with that of the primary oligomycin resistance (see Chapter 6) but also the cold lability of the resistance. There is therefore no evidence to support any suggestion that the thermosensitivity is due to a gene additional to that concerned in oligomycin resistance. One of the oligomycin

resistant mutants, D22A12, has, on occasion, shown in both liquid and solid medium, drug dependence, growth on non-fermentable medium being extremely poor in the absence of oligomycin. This mutant which seems in some measure to resemble the streptomycin-dependent mutants isolated from E.coli (Coukell and Polglase, 1970; Apirion and Schlessinger, 1967; Apirion et al., 1969) and Chlamydomonas reinhardii (Schimmer and Arnold, 1970) differs from them, in that the phenotype is only expressed irregularly and under conditions which are not yet fully defined. Further work is in progress on this mutant.

CHAPTER 6

THE GENETICS OF THE OLIGOMYCIN MUTANTS

Introduction

As recent reviews encompassing large areas of yeast cytoplasmic genetics (Bolotin et al., 1971 ; Coen et al., 1970 ; Preer, 1971 ; Linnane and Haslam, 1970) have been published and a summary of the cytoplasmic genetics and the genetics of yeast drug resistant mutants presented in chapter 1, this introduction is restricted solely to an analysis of the rationale underlying the type of genetic experiments undertaken. In this chapter data concerning the genetic systems governing oligomycin resistance in the two major phenotypic classes of mutants described in chapters 4 and 5 are presented. The temptation to work on a restricted number of mutants has been resisted and an attempt made to see if there is a clear division between the type of genetics shown by the two phenotypic classes of resistant mutants. In particular, attention has been paid to whether the mutants exhibited cytoplasmic or nuclear inheritance. In the former case attempts were made to correlate the determinant with mitochondrial DNA. On the naive assumption that 'mitochondrial DNA make mitochondrial RNA makes mitochondrial protein' (Vesco and Penman, 1971 ; Surzycki and Gillham, 1971) this would provide independent evidence as to the site of oligomycin resistance.

In yeast, genes for drug resistance that are cytoplasmically inherited show the following characteristics (Coen et al., 1970 ; Roodyn and Wilkie, 1968 ; Saunders et al., 1971 ; Bunn et al., 1970) :

(i) Mitotic segregation for resistance and sensitivity amongst the diploids from resistant ρ^+ x sensitive ρ^+ crosses.

(ii) Lack of such segregation in diploids from resistant ρ^+ x sensitive ρ^- crosses.

(iii) Loss of resistance in many cases on formation of ρ^- cells from a resistant ρ^+ strain.

(iv) Segregation of spores showing either 4 :0 or 0 :4 resistant : sensitive ratios from tetrads of resistant and sensitive diploids respectively.

The initial experiments attempt to define, by using these criteria and others, the mode of inheritance of the various mutants isolated. Investigation of the allelic

relationship of the various mutants in each phenotypic class have also been undertaken, both to estimate the minimal number of determinants which can confer oligomycin resistance on the cell, and to provide a guide as to the strains which were worthy of further biochemical analysis. The studies of the class II mutants have extended to attempts to map the various non allelic groups and to determine their relationship to the cytoplasmic genes concerned in chloramphenicol and erythromycin resistance.

As biochemical analysis of the class I mutants has shown that these mutants also appear to express their oligomycin resistance at the mitochondrial level (J. R. Turner, private communication; Griffiths et al., 1972), studies on these mutants have been extended in an attempt to understand the many puzzling features exhibited by their genetics (see this chapter). The importance of understanding these mutants is increased by the cryosensitivity of oligomycin resistance in many of these strains (chapter 5).

Results

Oligomycin resistance in diploids derived from resistant haploids by OL^R x OL^S crosses

Qualitative analysis

Oligomycin resistant haploids were mass mated to a ρ^+ sensitive tester of the opposite mating type (chapter 2) and the resulting diploids of these $OL^R\ \rho^+$ x $OL^S\ \rho^+$ crosses purified of any contaminating haploids by restreaking onto MMGlu plates. They were then tested for their level of oligomycin resistance by dropping out random selections of about 1000 diploids of each strain onto YEPGly plates containing various levels of oligomycin and incubated at 30°C for three days when the plates were scored. The results are shown in Table 6.1.

The diploids obtained showed widely varying levels of resistance, in agreement with the findings of Stuart (Stuart, 1970), some showing levels of oligomycin resistance as high as that of the haploid strain from which they were derived (e.g. D22A18 and D6A15) whilst others showed resistance intermediate between those of the parental strains involved in the cross or as low as that of the sensitive tester strain used. Examination of Table 6.1. shows that there is a correlation between the division into classes made on phenotypic grounds (chapter 4) and the level of resistance shown by the diploids derived from these

TABLE 6.1.

Level of Oligomycin Resistance Exhibited by Diploids from $OL^{R}_{\rho}{}^{+}$ x $OL^{S}_{\rho}{}^{+}$ Crosses

Diploids from $OL^{R}_{\rho}{}^{+}$ x $OL^{S}_{\rho}{}^{+}$ crosses were obtained by the mass mating technique described in chapter 2. The resulting diploids were restreaked onto MMGlu plates once, incubated at 30°C for 3 days, when random cell selections were tested for oligomycin resistance by dropping out onto YEPGly drug plates. The concentrations of oligomycin used were 0, 0.5, 1.0, 2.5, 5.0 and 10.0 γ/ml. Scoring was carried out after 3 days incubation at 30°C.

TABLE 6.1.

Cross	Class of OL^R Mutant	Oligomycin Resistance of Haploid	Oligomycin Resistance of Diploid
D22 A1 x D6	III	2.5	-
D22 A2 x D6	I	2.5	-
D22 A3 x D6	I	>10.0	1.0
D22 A4 x D6	I	>10.0	1.0 - 2.5
D22 A5 x D6	I	>10.0	1.0
D22 A6 x D6	-	5.0	1.0
D22 A7 x D6	I	>10.0	1.0 - 2.5
D22 A8 x D6	-	>10.0	1.0 - 2.5
D22 A9 x D6	I	5.0	1.0
D22 A11 x D6	I	2.5	1.0
D22 A12 x D6	I	>10.0	2.5
D22 A13 x D6	II	>10.0	>10.0
D22 A14 x D6	II	>10.0	>10.0
D22 A15 x D6	II	>10.0	>10.0
D22 A16 x D6	II	>10.0	>10.0
D22 A18 x D6	II	2.5	2.5
D22 A19 x D6	II	>10.0	>10.0
D22 A20 x D6	I	10.0	2.5
D22 A21 x D6	II	>10.0	>10.0
D22 A22 x D6	II	>10.0	>10.0
D22 B1 x D6	II	>10.0	>10.0
D22 B2 x D6	-	>10.0	5.0
D22 B3 x D6	I	>10.0	2.5
D22 B4 x D6	I	>10.0	1.0
D22 B5 x D6	-	>10.0	1.0
D22 B6 x D6	I	>10.0	1.0 - 2.5

continued .....

TABLE 6.1. (continued)

Cross	Class of OL^R Mutant	Oligomycin Resistance of Haploid	Oligomycin Resistance of Diploid
D22 B8 x D6	I	>10.0	1.0
D22 B9 x D6	I	>10.0	1.0
D22 B10 x D6	I	5.0	1.0
D22 B13 x D6	-	10.0	2.5 - 5.0
D22 B15 x D6	I	>10.0	1.0
D22 B16 x D6	I	>10.0	1.0
D22 B19 x D6	-	>10.0	2.5
D22 B20 x D6	I	5.0	1.0
D22 B21 x D6	II	>10.0	>10.0
D22 B22 x D6	I	2.5	1.0
D22 B23 x D6	II	>10.0	>10.0
D22 C1 x D6	I	>10.0	2.5
D22 C2 x D6	II	>10.0	>10.0
D22 C3 x D6	-	-	0.5
D22 C4 x D6	II	>10.0	>10.0
D6 A1 x D22	II	>10.0	>10.0
D6 A3 x D22	I	2.5	1.0
D6 A4 x D22	I	10.0	-
D6 A5 x D22	II	>10.0	>10.0
D6 A9 x D22	I	>10.0	2.5
D6 A10 x D22	I	2.5	1.0 - 2.5
D6 A11 x D22	I	2.5	1.0 - 2.5
D6 A15 x D22	I	2.5	2.5
D6 B1 x D22	II	>10.0	>10.0
D6 B2 x D22	II	>10.0	>10.0
D6 B3 x D22	II	>10.0	>10.0

resistant strains. The class II mutants appear to give rise to diploids from $OL^{R}\rho^{+} \times OL^{S}\rho^{+}$ crosses which, within the limits of the assay system used, are as resistant as the haploid resistant strain from which they were derived - a result possibly compatible either with a cytoplasmic non-mendelian type of inheritance (Coen et al., 1970), or alternatively, with mendelian inheritance caused by a dominant gene. These alternatives are resolvable by various means and will be described below. The diploids from the class I mutants, on the other hand, always show levels of oligomycin resistance that are low or intermediate in comparison to the resistant haploids from which they were derived. Such results are explainable by mendelian inheritance by a gene(s) showing partial dominance or possibly some non-mendelian system, but not of the type occurring in cytoplasmic erythromycin or chloramphenicol resistant mutants, where the resistance if transmitted to the diploid cell should be as great as that shown by the haploid resistant strain.

Mitotic segregation in diploids derived from $OL^{R}\rho^{+} \times OL^{S}\rho^{+}$ crosses

As one of the characteristics of the cytoplasmic systems in yeast so far investigated has been mitotic segregation of different cell types from a cross in which all the diploids should have an identical nuclear genetic constitution (Coen et al., 1970), experiments were carried out to characterise further the mode of inheritance of oligomycin resistance within the class I and class II mutants. Attempts at quantification of the segregation patterns observed were undertaken using both replica and aliquot plating. The procedures used in both types of analysis are shown diagramatically in Figure 2.1.

(i) Quantitative Replica Plating

Diploids from $OL^{R}\rho^{+} \times OL^{S}\rho^{+}$ crosses involving both class I and class II mutants were obtained by mass mating and prototrophic selection (chapter 2). They were then respread onto MMGlu plates to isolate single colonies and after incubation at 30°C for three days, these colonies were replicated onto YEPGly plates $\pm$ oligomycin. The plates were scored for total and oligomycin resistant colonies after a further three days at 30°C and the results are listed in Table 6.2. Diploids derived from both class I and class II mutants, whether derived from parental strain D6 or D22, showed signs of apparent mitotic segregation for

TABLE 6.2.

Segregation Patterns in Diploids from
$OL^{R}\rho^{+} \times OL^{S}\rho^{+}$ Crosses

ASSAY : REPLICA PLATING

Diploids from $OL^{R}\rho^{+} \times OL^{S}\rho^{+}$ crosses were obtained by mass mating and prototrophic selection. These diploids were spread onto MMGlu plates to isolate single colonies and, after 3 days incubation at 30°C, the plates were replicated onto YEPGly ± oligomycin.

The plates were scored for the total colony number as well as for oligomycin resistant, sensitive and mixed colonies, after a further 3 days at 30°C. Routinely 0 and 2.5 γ/ml oligomycin were used in the YEPGly plates, but in experiments on the dependence of diploid growth on oligomycin concentration, 1.25 and 5.0 γ/ml oligomycin plates were also used.

TABLE 6.2.

Cross	Genotype of Resistant Strain	Total No. of Cells Analysed	Number Resistant Colonies	Number Sensitive Colonies	Number Mixed Colonies	Percentage Transmission of OL Resistance
			CLASS I MUTANTS			
D22 A3 x D6	OL^RA3	23	0	0	23	-
D22 A5 x D6	OL^RA5	143	0	2	141	-
D22 A7 x D6	OL^RA7	31	0	0	31	-
D22 A12 x D6	OL^RA12	120	0	0	120	-
D22 A20 x D6	OL^RA20	339	1	0	338	-
D22 B10 x D6	OL^RB10	236	0	60	176	-
D22 B16 x D6	OL^RB16	24	0	0	24	-
D6 A4 x D22	OL^RA4/1	265	0	1	264	-
D6 A9 x D22	OL^RA9/1	46	0	0	46	-
D22 A20D6H1 x D22	OL^RA20	167	3	8	156	-

continued

TABLE 6.2. (continued)

Cross	Genotype of Resistant Strain	Total No. of Cells Analysed	Number Resistant Colonies	Number Sensitive Colonies	Number Mixed Colonies	Percentage Transmission of OL Resistance
			CLASS II MUTANTS			
D22 A13 x D6	OL^RA13	20	10	10	0	50.0
D22 A14 x D6	OL^RA14	271	190	81	0	70.0
D22 A15 x D6	OL^RA15	192	167	25	0	87.0
D22 A16 x D6	OL^RA16	634	567	67	0	88.5
D22 B1 x D6	OL^RB1	365	278	87	0	77.0
D22 C4 x D6	OL^RC4	554	307	247	0	55.0
D6 A1 x D22	OL^RA1/1	531	399	132	0	24.0
D6 A5 x D22	OL^RA5/1	332	100	232	0	29.0
D6 B2 x D22	OL^RB2/1	465	240	225	0	53.0
D6 B3 x D22	OL^RB3/1	526	32	494	0	8.0

continued

TABLE 6.2. (continued)

Cross	Genotype of Resistant Strain	Total No. of Cells Analysed	Number Resistant Colonies	Number Sensitive Colonies	Number Mixed Colonies	Percentage Transmission of OL Resistance
			CONTROLS			
D22 Er 310 x D6	Er^{R} 310	604	412	192	0	68.0
D22 x D6	-	352	0	0	0	0

oligomycin resistance. This behaviour is completely unlike that shown by either the adenine or arginine and methionine genetic markers which are present in these diploids and known to have nuclear inheritance. However, the actual form that the segregation took was very different, depending on whether the diploid was derived from an $OL^{R}\rho^{+}$ x $OL^{S}\rho^{+}$ cross involving a class I or a class II oligomycin resistant strain.

Diploids from class I crosses showed segregation extending to virtually all the colonies replicated, the oligomycin resistant growth occurring, mostly taking the form of mixed colonies showing growth varying from the markedly papillate to the visually confluent. This can be seen more clearly by reference to Plates 3 and 4. Very few of the diploid colonies from this type of cross appeared totally devoid of resistant growth and similarly, few colonies showed visually confluent growth. The amount of oligomycin resistant growth shown by these diploid colonies was highly dependent on the level of oligomycin in the growth medium used for replication - the higher the oligomycin level, the less resistant growth was present. This result corresponds neatly with the results shown in Table 6.1. as diploids from crosses of class I OL^{R} x OL^{S} were found to show low or intermediate levels of resistance, and the finding of diminishing growth with increasing oligomycin concentrations noted here would lead, when a standard inoculum was used, to exactly this result. It should be stressed that under these conditions of replication and growth, diploids derived from parental D6 x D22 crosses ($OL^{S}\rho^{+}$ x $OL^{S}\rho^{+}$) fail to show oligomycin resistant growth (Plate 3). No transmission values have been quoted in Table 6.2. for the class I mutants as, for reasons to be discussed later, the assumption that all colonies showing resistant or partially resistant oligomycin growth were derived from cells exhibiting resistance may be untrue and lead to false correlations between these class I mutants and known cytoplasmic systems. However, it is evident that most colonies show some resistant growth (Plate 3). In contrast to these results found on drug plates, none of the diploids derived from class I mutants show any peculiarity in the growth on YEPGly in the absence of the drug (Plates 3 and 4).

The picture provided by the class II mutants is entirely different. Diploids from crosses of $OL^{R}II\,\rho^{+}$ x $OL^{S}\rho^{+}$ show mitotic segregation identical to that shown by cytoplasmic erythromycin and

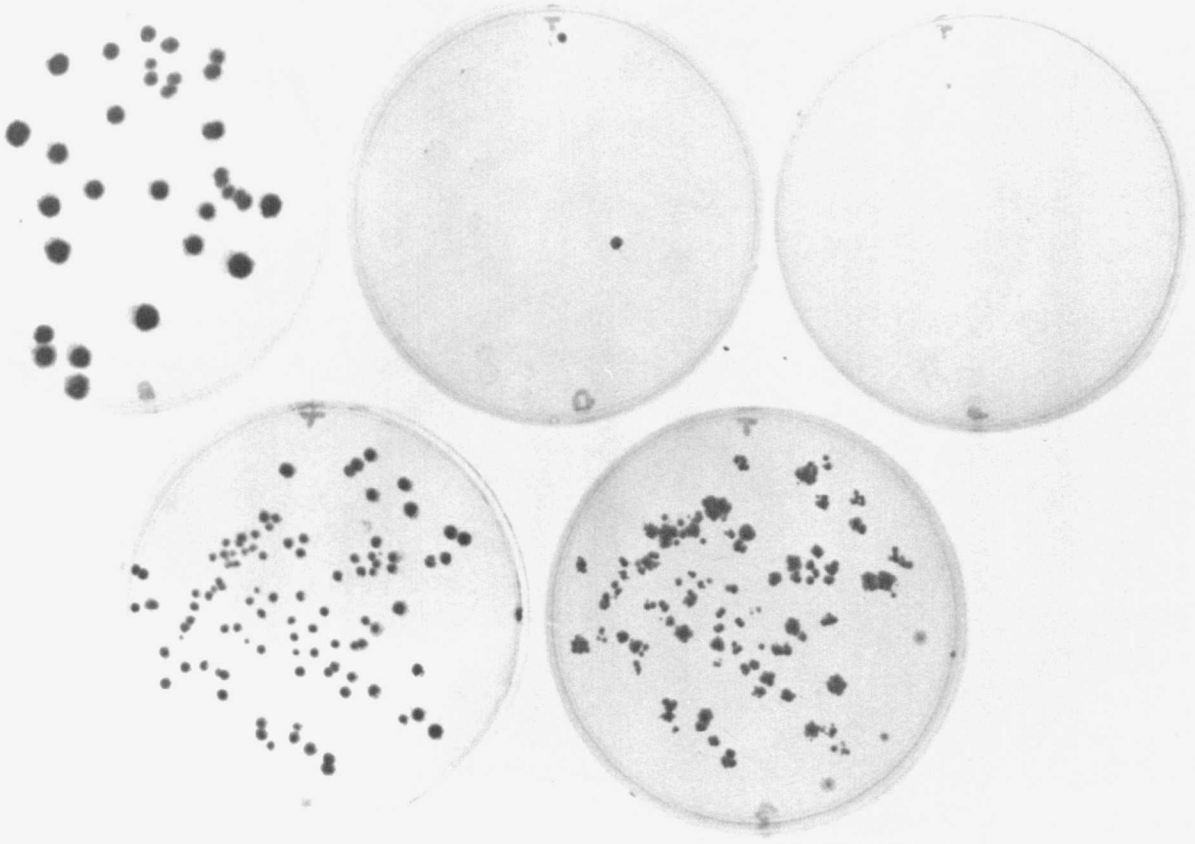


PLATE 3

Left to Right

Master Plate	YEPGly + 2.5 γ/ml Oligomycin	YEPGly + 5.0 γ/ml Oligomycin
D22 x D6 (OL^S x OL^S)	D22 x D6	D22 x D6
D22 A7 x D6 (OL^R I x OL^S)	--	D22 A7 x D6

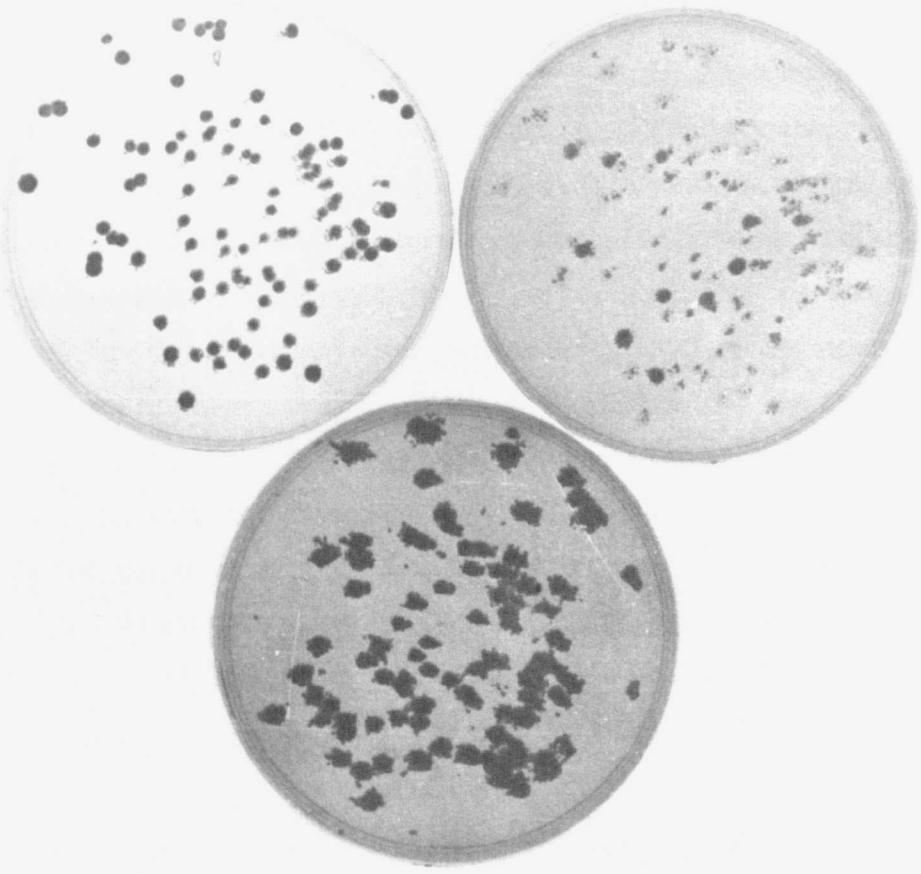


PLATE 4

Left to Right

D22 B9 x D6 (OL^R I x OL^S)
MMGlu

D22 B9 x D6
YEPGly + Oligomycin

D22 B9 x D6
YEPGly

chloramphenicol mutants (Coen et al., 1970; Bolotin et al., 1971) with resistant and sensitive colonies arising from the same cross. There was a complete absence of papillate growth on the colonies when grown in the presence of oligomycin, colonies appearing either totally resistant or completely sensitive. Again, unlike the results obtained with the diploids from class I mutants, no reduction in either the number of resistant colonies or the amount of growth such colonies made was seen when the level of oligomycin in the growth media was increased, providing, of course, that this level was not higher than the level of resistance shown by the haploid resistant mutant used in the cross. No abnormalities in glycerol supported growth were noted in these diploids and the transmission rates obtained with these oligomycin resistant mutants are within the same order as those shown by the cytoplasmic erythromycin resistant strains isolated from the same parental strain (Table 6.2). In this respect it must be noted that the class II mutants derived from parental strain D22 appear to show higher transmission of oligomycin resistance when crossed to D6 than do D6 OL^{R} II mutants when crossed to D22.

(ii) Quantitative Aliquot Plating

When diploids from a cross of class I mutants $OL^{R}\rho^{+}$ x $OL^{S}\rho^{+}$ were tested for mitotic segregation by quantitative aliquot plating, only a very small proportion of the viable respiratory competent cells showed oligomycin resistance, i.e. very low transmission values were apparent. The data are shown in Table 6.3. The number of oligomycin resistant diploid colonies appearing was dependent on the concentration of oligomycin in the growth media - less colonies apparently growing at high oligomycin concentrations (Table 6.3). This result is in agreement with those obtained during replica plating analysis. These results would seem to suggest that the effect of high concentrations of oligomycin on replica plating and dropping out experiments is not due to a uniform slowing of the growth of the whole diploid cell population but due to a selective effect against the majority of diploid progeny. The very low resistance transmission values found in diploids from $OL^{R}I\rho^{+}$ x $OL^{S}\rho^{+}$ crosses are not caused by spontaneous resistant mutants arising in the diploids as :

(i) The period of incubation before scoring is not long enough for such mutants to appear

(ii) Diploids of D22 x D6 fail to show any resistant mutant colonies in identical experiments to those carried out with the class I mutants

TABLE 6.3.

Transmission of Oligomycin Resistance in Diploids from $OL^{R}\rho^{+} \times OL^{S}\rho^{+}$ Crosses

ASSAY : ALIQUOT PLATING

Diploids from $OL^{R}\rho^{+} \times OL^{S}\rho^{+}$ crosses were obtained by mass mating and prototrophic selection at 30°C. After 4 - 6 days, suspensions of these diploids were prepared at various dilutions and spread onto MMGlu and MMGly plates ± oligomycin. From a comparison of the number of colonies growing in the presence and absence of oligomycin, transmission frequencies were calculated. Scoring was carried out after 6 - 8 days at 30°C. Oligomycin, when used, was added to the media at final concentrations of either 2.5 or 5.0 γ/ml.

Where more than one value is listed in the table, results are from separate crosses made more than six months apart (2 sub-culturings).

TABLE 6.3.

OL^R Strain Involved in Cross	Genotype of OL^R Strain	Percentage Transmission Oligomycin Resistance in Diploids	
		Assay : 2.5 γ/ml Oligomycin	Assay : 5.0 γ/ml Oligomycin
		CLASS I MUTANTS	
D6A4	OL^RA4/1	7.5×10^{-3}	2.5×10^{-3}
D6A9	OL^RA9/1	5.6×10^{-3}	1.8×10^{-3}
D22A5	OL^RA5	3.8×10^{-4}, 6.0×10^{-2}	$<7.0 \times 10^{-5}$, 3.0×10^{-3}
D22A7	OL^RA7	2.8×10^{-4}	-
D22A7D62	OL^RA7	7.0×10^{-4}	-
D22A7D611	OL^RA7	7.0×10^{-4}	-
D22A12	OL^RA12	1.0	-
D22B8	OL^RB8	3.0×10^{-4}	-
D22B8D6S1	OL^RB8	1.3×10^{-4}	-

continued

TABLE 6.3. (continued)

OL^R Strain Involved in Cross	Genotype of OL^R Strain	Percentage Transmission Oligomycin Resistance in Diploids	
		Assay : 2.5 γ/ml Oligomycin	Assay : 5.0 γ/ml Oligomycin
		CLASS I MUTANTS	
D22 B9	$OL^R B9$	7.0×10^{-4}, 6.0×10^{-2}, 4.0×10^{-5}	$< 9.0 \times 10^{-4}$
D22 B9 D6 C	$OL^R B9$	7.0×10^{-3}, 3.6×10^{-3}	-
D22 B10	$OL^R B10$	1.1, 2.5	1.1, 2.5
D22 B16	$OL^R B16$	6.0×10^{-5}	-
		CLASS II MUTANTS	
D22 A13	$OL^R A13$	73.0	73.0
D22 A16	$OL^R A16$	90.0, 68.0	90.0, 68.0
D22 A21	$OL^R A21$	53.0	53.0

continued

TABLE 6.3. (continued)

OL^R Strain Involved in Cross	Genotype of OL^R Strain	Percentage Transmission Oligomycin Resistance in Diploids	
		Assay : 2.5 γ/ml Oligomycin	Assay : 5.0 γ/ml Oligomycin
		CLASS II MUTANTS	
D6 A1	OL^R A1/1	26.5	26.5
D6 B1	OL^R B1/1	33.0	33.0
D6 B2	OL^R B2/1	54.0	-
D6 B3	OL^R B3/1	6.0	-
		CONTROL	
D22 x D6	-	$< 6.25 \times 10^{-6}$	-

(iii) The lowest transmission frequency shown by diploids from $OL^{R}I\rho^{+}$ x $OL^{S}\rho^{+}$ crosses is still some ten-fold greater than the highest values for spontaneous oligomycin resistance in diploids of D22 x D6 ($OL^{S}\rho^{+}$ x $OL^{S}\rho^{+}$) ever obtained

(iv) Experiments on kinetics of resistance loss (see page 96 demonstrate that this low resistance frequency is not due to a mutational event(s).

Two further facts of importance must be mentioned. Firstly, there is a good deal of variability found in the transmission values recorded from experiment to experiment for a single cross. Factors which seem to influence the value recorded include the length of the incubation period between plating out and scoring and the length of time from mating to the initial harvesting of the diploids. Secondly, the diploids from crosses of $OL^{R}I\rho^{+}$ x $OL^{S}\rho^{+}$ which were resistant to oligomycin on aliquot plating, showed a highly heterogeneous size distribution when grown in the presence of the drug. Grown in the absence of the drug, all colonies showed a highly uniform size distribution.

Comparison of Tables 6.2. and 6.3. shows that there is a large discrepancy between the transmission frequency found for oligomycin resistance when measured by aliquot plating and when measured by replica plating. For instance, whilst the oligomycin resistance frequency for diploids from a D22 A7 x D6 cross was estimated to be 2.8×10^{-4}% by aliquot plating, a value of 100% would have been derived from the replica plating experiments. This non-correlation of the replica and aliquot plating results has been analysed more stringently, and the results are shown in Table 6.4. In these experiments three class I OL^{R} mutants were mated proto trophically to a sensitive ρ^{+} tester and the diploids recovered after 4 - 6 days incubation at 30°C. Replica and aliquot analyses were carried out in parallel on the same suspensions of diploids. The results show decisively the lack of correlation between these two analyses.

Whilst most of the class I mutants show decreasing oligomycin resistance transmission with increasing levels of oligomycin, two of the mutants tested, D22 B10 and D22 A12 did not, and they also show transmission values that are higher than those shown by the majority of class I mutants. It therefore appears that within the class I mutants there is some genetic heterogeneity.

The frequency of transmission of oligomycin resistance in diploids from

TABLE 6.4.

Correlation of the Replica and Aliquot Plating Assay Results for Diploids from $OL^{R}\rho^{+}$ x $OL^{S}\rho^{+}$ Crosses Involving Class I or Class II Type Haploids

Diploids were obtained from $OL^{R}\rho^{+}$ x $OL^{S}\rho^{+}$ crosses by mass mating (chapter 2). After 4 - 6 days at 30°C, diploid cells were harvested and tested for their oligomycin resistance transmission by parallel replica and aliquot plating procedures. The drug plates used contained 2.5 γ/ml oligomycin.

Key

* Class I replica assay figures include mixed colonies - bracketed figures are for those colonies showing confluent resistant growth only - visibly mixed colonies excluded.

TABLE 6.4.

Oligomycin Resistant Strain	Cross	Percentage Transmission of Resistance Assay : Aliquot Plating	Percentage Transmission of Resistance Assay : Replica Plating
	CLASS I MUTANTS*		
D22 A7 (OL^RA7)	D22 A7 x D6	2.7×10^{-4}	100 (15)
D22 A12 (OL^RA12)	D22 A12 x D6	1.0	100 (20)
D22 B9 (OL^RB9)	D22 B9 x D6	6.6×10^{-4}	100 (7)
D22 A7 D6 11 (OL^RA7)	D22 A7 D6 11 x D22	7.2×10^{-4}	100 (10)
D22 A7 D6 2 (OL^RA7)	D22 A7 D6 2 x D22	7.3×10^{-4}	100 (100)
	CLASS II MUTANTS		
D6 B2 (OL^RB2/1	D6 B2 x D22	49.0	55
D6 B3 (OL^RB3/1)	D6 B3 x D22	6.0	9

$OL^R I\ \rho^+ \times OL^S\ \rho^+$ crosses appears identical whether the class I mutant is derived from strain D22 or strain D6. Thus, there is no reciprocal asymmetry such as is shown by mitochondrial genes in heterosexual crosses (Bolotin et al., 1971; Coen et al., 1970) or as seems to occur in D22 Ant^R x D6 Ant^S homosexual crosses (this chapter). The contrast between the results obtained with the class II mutants to those obtained with the class I strains occurs in respect of all characteristics. Class II mutants show high levels of resistance transmission in crosses of $OL^R II\ \rho^+ \times OL^S\ \rho^+$ and this level of transmission is independent of the concentration of oligomycin used in the drug plates. This latter result coincides with observations made during replica plating assays (Table 6.2) and assay of resistance levels amongst diploids (Table 6.1). Colony size of diploids is also completely homogeneous, whether the cells are grown in the presence or absence of oligomycin. Furthermore, diploids from crosses involving class II mutants show an excellent correlation between resistance transmission as measured by replica and aliquot plating. This is confirmed in Table 6.4. where the results of studies done in parallel on the same diploid suspensions are shown.

One added contrast to the results of the class I mutants is that class II mutants derived from D6 generally transmit resistance to fewer diploids when crossed to D22 than do D22 OL^R mutants when crossed by D6. This behaviour is not strictly analogous to the polarity effect as currently described by Slonimski (Bolotin et al., 1971) as later findings indicate that both D6 and D22 are ρ^+ strains. It does appear, however, that strain D6 has some nuclear modifying elements which are responsible for causing the asymmetry noted.

Analysis of diploids from crosses of the type
$OL^R\ \rho^+ \times OL^{S(o)}\ \rho^-$

A characteristic of the systems exhibiting cytoplasmic inheritance is the presence of mitotic segregation in crosses of the type $X^R\ \rho^+ \times X^S\ \rho^+$ (Coen et al., 1970). Such segregation does not occur normally in crosses where the sensitive tester is a petite. This behaviour is similarly a characteristic of cytoplasmic inheritance. ρ^- testers were obtained by treating growing YEPG cultures of D22 and D6 with 10.0 γ/ml ethidium bromide (chapter 2). Table 6.5. shows that the degree of suppressiveness exhibited by the various D22 and D6 derived ρ^- strains used as testers is very low, so one may assume that the degree of retention of mitochondrial

TABLE 6.5.

Analysis of the Suppressiveness of Petites Derived from Strains D6 and D22 by Ethidium Bromide Mutagenesis

Petites were isolated as described in chapter 2. These petites were mated to a ρ^+ tester of the opposite mating type and individual diploid colonies isolated on MMGlu plates. Their degree of suppressiveness was estimated by replica plating individual diploid colonies onto YEPG and YEPGly media. Plates were scored after 4 days at 30°C. D6 ρ^{-2}, D6 ρ^{-3} and D6 ρ^{-4} were all isolated from cells that had undergone two cycles of exposure to ethidium bromide prior to petite isolation.

TABLE 6.5.

Tester Strain	ρ^- Strain Involved in Cross	Total No. of Cells Analysed	Total No. of Petites	Percentage Suppressiveness
D6	D22 ρ^{-1}	1184	4	0.30
D22	D6 ρ^{-1}	226	0	<0.50
D22	D6 ρ^{-2}	661	0	<0.16
D22	D6 ρ^{-3}	791	0	<0.13
D22	D6 ρ^{-4}	693	0	<0.15

DNA is minimal (Linnane and Haslam, 1971 ; Nagley and Linnane, 1970; Michaelis et al., 1971). Diploids were obtained by mating both class I and class II mutants to these various ρ^- testers. The results obtained on analysing by replica plating diploids from these $OL^{R}\rho^{+} \times OL^{S(o)}\rho^{-}$ crosses are shown in Table 6.6. When class I mutants were the resistant strain in the above cross, the diploids, with the exception of strain D22A5, showed behaviour indistinguishable from that shown by diploids from $OL^{R}I\rho^{+} \times OL^{S}\rho^{+}$ crosses. The majority of the diploid colonies were composed of mixtures of resistant and sensitive cells or showed papillate resistant growth. Out of the class I mutants tested, D22A5 alone showed greater resistance transmission in diploids from $OL^{R}I\rho^{+} \times OL^{S(o)}\rho^{-}$ crosses than from $OL^{R}I\rho^{+} \times OL^{S}\rho^{+}$ crosses.

This failure to find increased transmission of resistance in diploids from $OL^{R}I\rho^{+} \times OL^{S(o)}\rho^{-}$ crosses could be due to the non-deletion of that part of the mDNA carrying the resistant determinant in the particular ρ^- tester used. In order to eliminate this possibility, the experiments were repeated using other D22 and D6 petite strains as testers, including $D6\rho^{-2}$, $D6\rho^{-3}$ and $D6\rho^{-4}$, these latter three strains having been isolated from cells that had undergone at least two successive growth periods in the presence of 10.0 γ/ml ethidium bromide and had then been isolated and characterised as ρ^-. This procedure was adopted to try and delete any mDNA which might remain in ρ^- strains such as $D6\rho^{-1}$, which had been isolated after only a single exposure to 10.0 γ/ml ethidium bromide. Table 6.7. shows that, in fact, the same results were obtained with diploids from $OL^{R}\rho^{+} \times OL^{S(o)}\rho^{-}$ crosses when these three strains were used as the $OL^{S(o)}\rho^{-}$ testers as had been obtained previously with $D6\rho^{-1}$ as tester strain. It therefore seems unlikely that the determinant for the class I type of oligomycin resistance is carried on mitochondrial DNA.

In contrast to these results, the class II mutants showed the behaviour expected of cytoplasmic mutants. Diploids from crosses of $OL^{R}II\rho^{+} \times OL^{S(o)}\rho^{-}$ were all resistant to oligomycin and there was a complete absence of mitotic segregation.

Petite induction in oligomycin resistant mutants

Cytoplasmic petite induction in S.cerevisiae has been associated with a loss of informational content from mitochondrial DNA either due to total deletion of the

TABLE 6.6.

Analysis of Segregation Patterns in Diploids from $OL^{R}\rho^{+} \times OL^{S}\rho^{-}$ Crosses

ASSAY : REPLICA PLATING

Oligomycin resistant mutants of class I and class II were mass mated to ρ^{-} oligomycin sensitive testers which had been isolated after petite induction with 10.0 γ/ml ethidium bromide (Table 6.5). Diploid colonies from these $OL^{R}\rho^{+} \times OL^{S(o)}\rho^{-}$ crosses were obtained on MMGlu plates and were analysed for mitotic segregation by replica plating onto YEPGly plates $\pm$ oligomycin. The plates were scored after incubation for 3 days at 30°C.

TABLE 6.6.

Cross	Genotype of OL^R Mutant	Total No. of Colonies	Total No. Resistant Colonies	Total No. Sensitive Colonies	Total No. Mixed Colonies	Percentage Transmission of Resistance
		Class I Mutants ρ^+ x Sensitive Tester ρ^-				
D22 A5 x D6 ρ^{-1}	OL^RA5	957	957	0	0	100
D22 A7 x D6 ρ^{-1}	OL^RA7	657	0	3	654	-
D22 A12 x D6 ρ^{-1}	OL^RA12	240	48	0	192	-
D22 B9 x D6 ρ^{-1}	OL^RB9	246	0	42	204	-
D6 A4 x D22 ρ^{-1}	OL^RA4/1	226	7	1	218	-
D6 A9 x D22 ρ^{-1}	OL^RA9/1	150	0	0	150	-
D6 A15 x D22 ρ^{-1}	OL^RA15/1	530	2	0	528	-
		Class II Mutants ρ^+ x Sensitive Tester ρ^-				
D22 A13 x D6 ρ^{-1}	OL^RA13	33	33	0	0	100
D22 A16 x D6 ρ^{-1}	OL^RA16	729	729	0	0	100

TABLE 6.7.

Transmission of Oligomycin Resistance in Diploids from $OL^{R}I\rho^{+}$ x $OL^{S(o)}\rho^{-}$ Crosses

ASSAY : REPLICA PLATING

D22 $OL^{R}I\rho^{+}$ strains were mass mated to D6 $OL^{S(o)}\rho^{-}$ strains isolated from cells that had undergone two or three cycles of exposure to 10.0 γ/ml ethidium bromide (chapter 2). The diploids were analysed for oligomycin resistance by replica plating onto YEPGly $\pm$ 2.5 γ/ml oligomycin. Scoring was carried out after 3 days incubation at 30°C.

TABLE 6.7.

Cross	Genotype of OL^R Mutant	Total No. of Colonies Analysed	Total No. OL^S Colonies	Total No. OL^R Colonies	Total No. Mixed ($OL^S + OL^R$) Colonies	Percentage Transmission of Oligomycin Resistance
D22 A5 x D6 ρ^{-3}	OL^RA5	57	0	57	0	-
D22 A7 x D6 ρ^{-3}	OL^RA7	97	0	0	97	-
D22 A7 x D6 ρ^{-4}	OL^RA7	131	0	0	131	-
D22 A12 x D6 ρ^{-2}	OL^RA12	105	0	0	105	-
D22 A12 x D6 ρ^{-4}	OL^RA12	65	0	4	61	-
D22 A12 x D6 ρ^{-5}	OL^RA12	150	0	31	119	-
D22 B9 x D6 ρ^{-2}	OL^RB9	51	0	0	51	-
D22 B9 x D6 ρ^{-3}	OL^RB9	170	0	0	170	-
D22 B9 x D6 ρ^{-4}	OL^RB9	93	0	0	93	-
			CONTROLS			
D22 B9 x D6	OL^RB9	80	0	0	80	-
D22 A12 x D6	OL^RA12	69	0	0	69	-

mDNA (neutral petites) (Michaelis et al., 1971 ; Nagley and Linnane, 1970) or due to partial loss and alteration in the base composition of the remaining DNA (Bernardi et al., 1968 ; Carnevali et al., 1969). Studies with erythromycin resistant mutants have shown that this cytoplasmic resistance is lost on petite induction, allowing correlation and localisation of the gene conferring resistance with mDNA (Thomas and Wilkie, 1969, a, b). Although later work by Linnane and Slonimski's group has shown that loss of resistance is not obligatory in all cases of petite induction (Gingold et al., 1969; Deutsch et al., 1971), the converse that where resistance is lost on petite formation the gene is localised on mDNA, has not been doubted. This section describes experiments designed to test whether oligomycin resistance in the class I and II mutants was lost on formation of petites from the original grande cell lines. The petites were induced as described in chapter 2, using 2.5 γ/ml euflavine. As it is impossible to test the resistance of the petites directly as they are unable to grow on YEPGly media, the petites were mated to $OL^{S}\rho^{+}$ tester strains and the oligomycin resistance of the diploids tested. As any resistance in the diploids must be transmitted by the petite derived from the original resistant grande cell line, it is possible to discover whether petite formation has caused the loss of the oligomycin resistant determinant or not. In most cases three to five petites were isolated and analysed for each strain examined. Preliminary experiments with the class I mutants involved dropping out diploids from crosses of the type $OL^{R}(o)\rho^{-}$ x $OL^{S}\rho^{+}$ and $OL^{R}\rho^{+}$ x $OL^{S}\rho^{+}$ onto YEPGly plates $\pm$ oligomycin, and observing whether cells from the former crosses were all oligomycin sensitive. The latter crosses acted as controls.

This analysis did not permit of a clear answer. Diploids from crosses involving both the petites and the original resistant grande strains showed some resistance to oligomycin, but the amount of resistant growth observed depended on the level of oligomycin in the medium. For instance, diploids from crosses involving D6A4, D22A4, D22A7, D22A11 and D22B15 and diploids of the derived petites, all showed confluent growth on media containing 0, 0.5, 1.0 γ/ml oligomycin whilst in the presence of 2.5 γ/ml oligomycin non-confluent growth was apparent. 5.0 and 10.0 γ/ml oligomycin caused the virtual cessation of cell growth. Although it was apparent therefore that petite formation did not cause a total loss of resistance in the class I mutants, it was impossible to distinguish

whether partial losses of resistance such as Gingold et al. have described were occurring (Gingold et al., 1969). Later analyses of both the class I and class II mutants were therefore performed using an aliquot plating approach. The results are shown in Table 6.8.

Transmission of oligomycin resistance amongst the diploid progeny of crosses of $OL^{R}I\rho^{-}$ x $OL^{S}\rho^{+}$ is very low, but no smaller than that found already amongst the progeny of $OL^{R}I\rho^{+}$ x $OL^{S}\rho^{+}$ crosses. Considering the apparent instability characteristic of diploids from crosses involving class I mutants, the results are in good agreement, suggesting therefore that petite formation does not involve any loss in the oligomycin resistant determinant. This interpretation is supported by the finding that the transmission of resistance in diploids from $OL^{R}I\rho^{-}$ x $OL^{S}\rho^{+}$, though low, is still well above the spontaneous mutational background level. Further analysis of these class I petites would only be profitable if carried out using diploids that have undergone few divisions since mating. In this case the transmission values obtained from the control crosses $OL^{R}I\rho^{+}$ x $OL^{S}\rho^{+}$ are much higher and therefore any alteration of transmissability in the petite strains will be more noticeable.

The analysis of the class II mutants and their derived petites shows that strains D22A15 and D22A18 lose resistance on petite induction with euflavine. Whilst all the petites from the former strain have lost resistance, in the latter case one of the petites has completely lost resistance and one only partially. The oligomycin resistant determinants appear, therefore, to be localised on mitochondrial DNA in these class II strains. D22A21 on the other hand was not found to lose its resistance on petite induction with euflavine. This does not necessarily imply that the determinant is not localised on mDNA (Gingold et al., 1969; see rest of chapter), but may imply that if mitochondrially located, it is at a different locus from the determinants carried by strains D22A15 and D22A18 and less easily deleted by euflavine. The small differences in transmission frequency shown by D22A21 and the petites isolated from it are not thought significant and may either be due to sampling error or to differences in the transmissability of given markers in ρ^{+} and ρ^{-} cells (Slonimski, private communication).

The difference in behaviour of D22A21 from D22A15 and D22A18 is in accord with the results of recombination-allelism and three point mapping studies

TABLE 6.8.

Oligomycin Resistance Transmission Amongst Diploid Progeny of $OL^{R}\rho^{-}$ x $OL^{S}\rho^{+}$ Crosses

ASSAY : ALIQUOT PLATING

Class I and Class II mutants and the petites isolated from them after treatment with 2.5 γ/ml euflavine were mass mated to an $OL^{S}\rho^{+}$ tester and the resulting diploids tested by aliquot plating for the degree of transmission of oligomycin resistance. Plates were scored after 6 - 8 days incubation at 30°C. The levels of oligomycin in the YEPGly media used for aliquot plating were 0 and 2.5 γ/ml.

* Where two values are given these are the results of separate crosses.

TABLE 6.8.

Cross	Class of OL^R Mutants	Percentage Transmission of Oligomycin Resistance in Diploids
D22 A5 x D6	I	3.44×10^{-3}
D22 A5 P1 x D6	I	3.46×10^{-4}
D22 A5 P3 x D6	I	1.15×10^{-4}
D22 A5 P4 x D6	I	$< 1.0 \times 10^{-4}$
D22 A7 x D6	I	2.1×10^{-2}, 2.2×10^{-1}
D22 A7 P1 x D6	I	1.1×10^{-2}, 1.2×10^{-1}
D22 A7 P3 x D6	I	2.9×10^{-2}, 2.5×10^{-1}
D22 A7 P4 x D6	I	7.3×10^{-2}, 1.0×10^{-1}
D22 B9 x D6	I	5.7×10^{-2}
D22 B9 P2 x D6	I	5.1×10^{-2}
D22 B9 P3 x D6	I	7.9×10^{-2}
D22 A15 x D6	II	36.0, 28.7
D22 A15 P1 x D6	II	$< 1.0 \times 10^{-4}$
D22 A15 P3 x D6	II	$< 1 \times 10^{-5}$, $< 1.4 \times 10^{-3}$
D22 A15 P4 x D6	II	$< 5 \times 10^{-4}$, $< 5.7 \times 10^{-3}$
D22 A18 x D6	II	50.0
D22 A18 P1 x D6	II	1.0
D22 A18 P3 x D6	II	$< 1.5 \times 10^{-4}$
D22 A21 x D6	II	40.0
D22 A21 P1 x D6	II	20.0
D22 A21 P2 x D6	II	15.0
D22 x D6		$< 6.25 \times 10^{-6}$

which similarly suggest that the genetic determinants in these strains are not identical.

Tetrad Analyses

Tetrads of strains representative of both the class I and class II mutants have been analysed as described in chapter 2. The results (Table 6.9) corroborate the conclusion that the class II OL^R mutants show cytoplasmic inheritance, as on dissection of tetrads from OL^R diploids obtained by crossing D22 A13 x D6 (OL^RII x OL^S), typically cytoplasmic segregation ratios of 4 :0 resistant : sensitive spores were found (Roodyn and Wilkie, 1968; Coen et al., 1970). The converse segregation ratio of 4 :0 sensitive : resistant spores has been obtained in tetrads of sensitive diploids from the same cross.

Tetrad analysis of the class I mutant D22 B9, on the other hand, indicates a normal pattern of mendelian inheritance as diploids from a cross of D22 B9 x D6 (OL^RI x OL^S) when sporulated gave tetrads containing two sensitive and two resistant spores. Although the number of tetrads analysed so far is extremely small, the result appears reproducible.

Random spore analysis of spores from the same cross, D22 B9 x D6, gave equal numbers of resistant to sensitive spores. This result supports the conclusion that a nuclear gene is concerned in the resistance of the class I mutants, though as the diploids are apparently unstable and will presumably segregate out 'resistant' and 'sensitive' cells (page 96) during the presporulation phase on non-selective media, the result is perhaps less meaningful than appears at first sight.

Analysis of OL^RI x OL^RII crosses

When diploids were obtained from class I ρ^+ x class II ρ^+ mutant crosses and the diploids tested for resistance by replica plating colonies on to YEPGly + 2.5 γ/ml oligomycin, all the crosses tested appeared to show behaviour closely similar, if not identical, to that shown by the diploids derived from crosses of class I mutants with a sensitive tester. The results are shown in Table 6.10. Plates 5 and 6 illustrate typical examples of the plating results observed. This behaviour, with segregation of resistant, mixed and sensitive colonies from a single cross, was exhibited regardless of which of the mutants in the cross was derived from parental strain D6 and which from strain D22. To some extent one would expect this phenomena to be masked where the cytoplasmically inherited class II oligomycin

TABLE 6.9.

Tetrad Analysis

Diploid Cross Sporulated	Phenotype of Colony Sporulated	No. of Tetrads with Segregation Ratios of : R : S 4 : 0	R : S 3 : 1	R : S 2 : 2	R : S 1 : 3	R : S 0 : 4	ad_2^+ / ad_2 2 : 2	arg^+ / arg 2 : 2	met^+ / met 2 : 2
D22 A13 x D6 (OL^RA13 x OL^S)	OL^R	3	-	-	-	-	3	3	3
D22 A13 x D6 (OL^RA13 x OL^S)	OL^R	18 spores		Random Spores		18 : 0	Resistant : Sensitive		
D22 B9 x D6 (OL^RB9 x OL^S)	Mixed ?	-	-	3	-	-	3	3	3
D22 B9 x D6 (OL^RB9 x OL^S)	Mixed ?	14 spores		Random Spores		7 : 7	Resistant : Sensitive		

TABLE 6.10.

Analysis of Diploids from $OL^{R}I\rho^{+}$ x $OL^{R}II\rho^{+}$ Crosses

ASSAY : REPLICA PLATING

Diploids of $OL^{R}I\rho^{+}$ x $OL^{R}II\rho^{+}$ crosses were obtained by mass mating and prototrophic selection on MMGlu medium. The diploids were spread onto MMGlu plates and incubated at 30°C prior to replicating onto YEPGly plates $\pm$ 2.5 γ/ml oligomycin. Scoring of the crosses was performed after 2 - 3 days incubation at 30°C.

TABLE 6.10

Cross	Genotypes of Strains	Percentage Mixed Colonies ($OL^S + OL^R$)	Percentage OL^R Colonies	Percentage OL^S Colonies	Total ρ^+ Colonies Analysed
D6 B1 x D22 A2	OL^RB1/1 x OL^RA2	77.4	20.8	1.8	390
D6 B1 x D22 A3	OL^RB1/1 x OL^RA3	61.0	39.0	0	448
D6 B1 x D22 A4	OL^RB1/1 x OL^RA4	59.4	54.8	1.0	655
D6 B1 x D22 A7	OL^RB1/1 x OL^RA7	63.0	37.0	0	489
D6 B1 x D22 A20	OL^RB1/1 x OL^RA20	53.7	46.3	0	95
D6 B1 x D22 B9	OL^RB1/1 x OL^RB9	70.6	29.4	0	228
D6 B1 x D22 B15	OL^RB1/1 x OL^RB15	64.4	35.4	0	379
D6 A1 x D22 A2	OL^RA1/1 x OL^RA2	71.8	24.0	4.2	71
D6 A1 x D22 A4	OL^RA1/1 x OL^RA4	26.4	41.0	32.5	178
D6 A1 x D22 A5	OL^RA1/1 x OL^RA5	63.6	36.5	0	49
D6 A1 x D22 A7	OL^RA1/1 x OL^RA7	72.9	27.1	0	155
D6 A1 x D22 B8	OL^RA1/1 x OL^RB8	76.4	19.9	3.7	432
D6 A1 x D22 B9	OL^RA1/1 x OL^RB9	70.8	29.2	0	432

TABLE 6.10. (continued)

Cross	Genotypes of Strains	Percentage Mixed Colonies ($OL^S + OL^R$)	Percentage OL^R Colonies	Percentage OL^S Colonies	Total ρ^+ Colonies Analysed
D6A1 x D22B10	OL^RA1/1 x OL^RB10	69.4	30.6	0	483
D6A1 x D22B15	OL^RA1/1 x OL^RB15	67.8	32.2	0	205
D22A21 x D6A9	OL^RA21 x OL^RA9/1	100.0	0	0	213
D22A21 x D22A7D62	OL^RA21 x OL^RA7	61.0	38.9	0	249
D22A21 x D22B8D6C1	OL^RA21 x OL^RB8	52.2	41.8	6.0	67
D22A21 x D22B9D6C	OL^RA21 x OL^RB9	16.8	83.2	0	173
D22A15 x D22A7D62	OL^RA15 x OL^RA7	36.9	55.6	7.5	306
D22A15 x D22A12D62	OL^RA15 x OL^RA12	60.3	37.9	1.9	486
D22A15 x D22B8D69	OL^RA15 x OL^RB8	3.2	38.7	58.1	186
D22A18 x D6A4	OL^RA18 x OL^RA4/1	24.0	72.7	3.3	150
D22A18 x D22B9D6C	OL^RA18 x OL^RB9	55.8	29.1	15.1	285
D22A18 x D22A7D62	OL^RA18 x OL^RA7	4.2	62.5	33.3	24

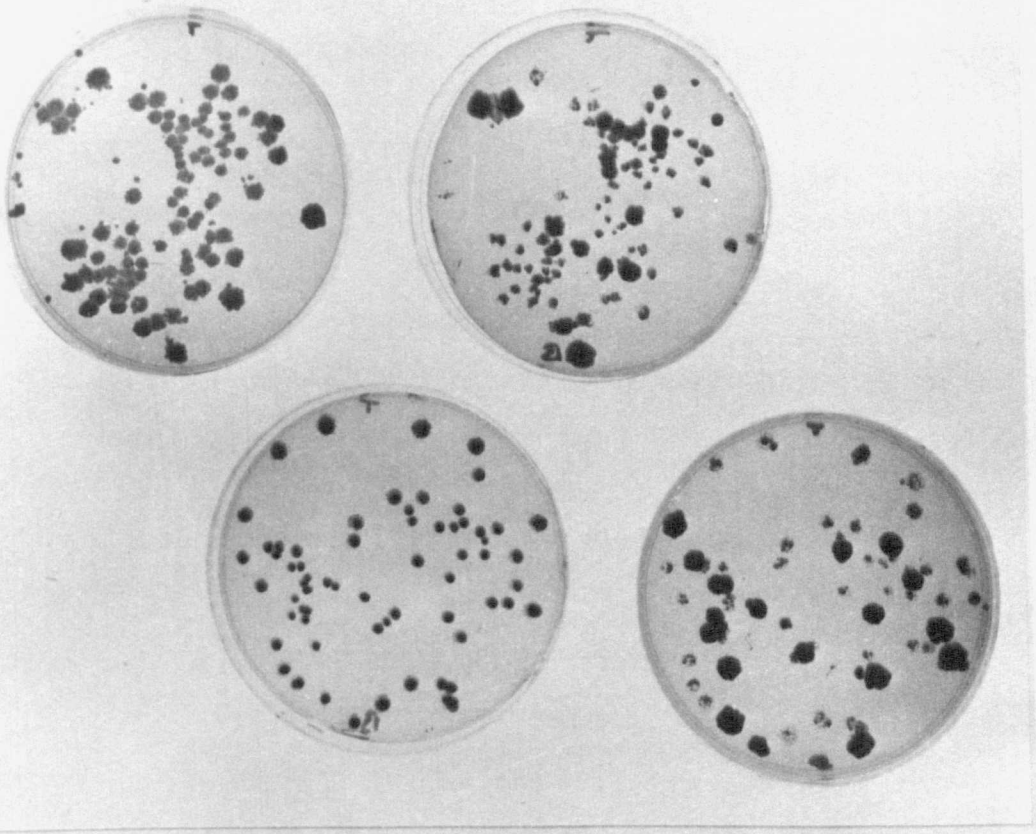


PLATE 5

Left to Right

D6 A1 x D22 A7 (OL^R II x OL^R I)
YEPGly

D6 A1 x D22 A7 (OL^R II x OL^R I)
YEPGly + Oligomycin

D6 A1 x D22 A4 (OL^R II x OL^R I)
YEPGly

D6 A1 x D22 A4 (OL^R II x OL^R I)
YEPGly + Oligomycin

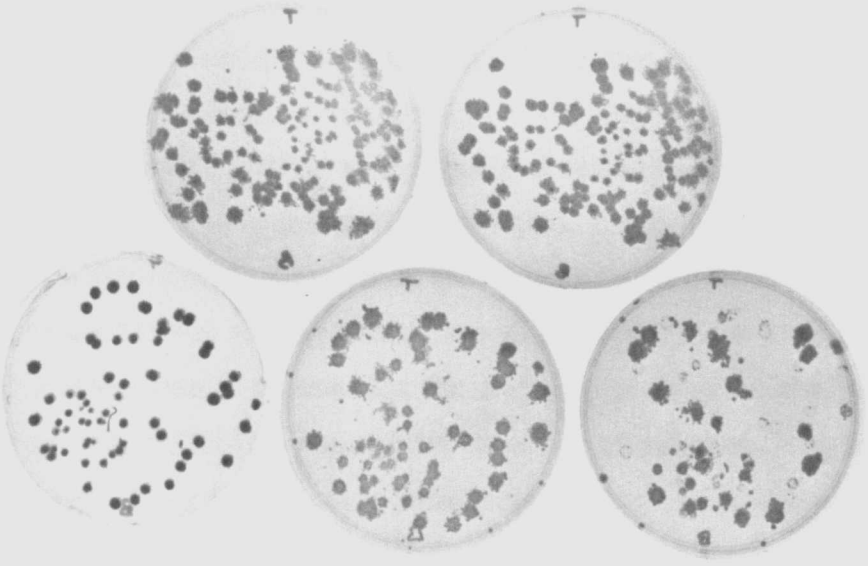


PLATE 6

Left to Right

D6 A9 x D22 B15 (OL^RI x OL^RI)
YEPGly

D6 A9 x D22 B15
YEPGly + Oligomycin

D22 A21 x D22 B8 D6 C1 (OL^RII x OL^RI)
MMGlu

D22 A21 x D22 B8 D6 C1
YEPGly

D22 A21 x D22 B8 D6 C1
YEPGly + Oligomycin

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ADDENDUM.

The discussion in the following section is based on a postulated model of inheritance for the class I mutants involving a cytoplasmic determinant distinct from the rho factor but which like rho is present in the haploid state in stable diploids. Thus segregation of OL^S cell types from $OL^R \times OL^R$ crosses will occur by recombination and segregation.

If alternatively, the oligomycin resistance is due solely to a nuclear determinant or due to a cytoplasmic determinant present in the diploid state in stable diploids then this segregation of oligomycin sensitive diploids should be understood to represent complementation rather than recombination. The use of the word 'recombination' throughout this section rather than 'complementation' is not meant to imply any unambiguously determined mechanism for the production of OL^S cell types in these crosses.

resistance determinant is highly transmitted to the diploid, but this was not observed with these crosses. The somewhat arbitrary nature of the division into sensitive, mixed and resistant colonies must be stressed as the so-called resistant colonies were resitant only visually whilst the mixed colonies were visibly mixtures of resistant and non-resistant cells, implying that they contain less than 10% resistant cells. The sensitive colonies were those that failed to show any visible papillae. As the percentage of resistant cells in a diploid cross is variable and apparently dependent on the time elapsing since the cross was made and the concentration of oligomycin used in the plating media (see this chapter), the numerical values have little value other than to stress that the majority of colonies always showed some papillate growth whilst in a substantial minority of colonies, this resistant growth was confluent. Very few colonies were wholly devoid of papillate resistant growth.

When diploids from $OL^{R}I\rho^{+}$ x $OL^{R}II\rho^{+}$ crosses were dropped out on to YEPGly plates containing 0, 2.5, 5.0 and 10.0 γ/ml oligomycin, the diploids appear to have only low resistance to oligomycin, a result analogous to that found with diploids from $OL^{R}I\rho^{+}$ x $OL^{S}\rho^{+}$ crosses. These results are shown in Table 6.11. which also shows, in agreement with the results in the following sections, that diploids from an $OL^{R}II\rho^{+}$ x $OL^{R}II\rho^{+}$ cross or an $OL^{R}I\rho^{+}$ x class I $OL^{R}I\rho^{+}$ cross do not exhibit this behaviour, showing resistance as high as that of the haploids from which they were derived.

All these results support the concept that the genetic loci governing oligomycin resistance in the class I and class II mutants are entirely different. The subdivision of the oligomycin resistant mutants into two broad phenotypic classes on the basis

ADDENDUM.

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All these results support the concept that the genetic loci governing oligomycin resistance in the class I and class II mutants are entirely different. The subdivision of the oligomycin resistant mutants into two broad phenotypic classes on the basis of cross resistance studies is therefore apparently a true reflection of the genetic differences.

Allelism-recombination analysis of class I OL^{R} mutants

Table 6.12 and Plate 6 show the results obtained when diploid progeny from various class I $OL^{R}\rho^{+}$ x class I $OL^{R}\rho^{+}$ crosses were analysed for oligomycin resistance The diploids obtained as described in chapter 2 were spread, replicated and scored as described in the legend to Table 6.12. It was hoped that two categories of diploid crosses would be observed - those giving only oligomycin resistant diploids, and those giving the mixture of sensitive, mixed and oligomycin resistant colonies, characteristic of diploids from crosses of the type $OL^{R}I\rho^{+}$ x $OL^{S}\rho^{+}$. In this case

TABLE 6.11.

Level of Oligomycin Resistance of Diploids from $OL^{R}I\rho^{+} \times OL^{R}II\rho^{+}$, $OL^{R}I\rho^{+} \times OL^{R}I\rho^{+}$, $OL^{R}II\rho^{+} \times OL^{R}II\rho^{+}$ Crosses

Diploids were obtained by the standard mass mating procedure. These diploids were purified by streaking onto MMGlu plates and then tested for their oligomycin resistance by dropping out onto YEPGly medium containing 0, 1.25, 2.5, 5.0 and 10.0 γ/ml oligomycin. Plates were scored after 3 days incubation at 30^{o}C.

TABLE 6.11.

Cross	Genotypes of Strains	Classes of Mutant Crossed	Oligomycin Resistance of Haploids γ/ml	Oligomycin Resistance of Diploids γ/ml
D6 B3 x D22 A1	OL^RB3/1 x OL^RA1	II x III	10.0, 2.5	0.5
D6 B3 x D22 A4	OL^RB3/1 x OL^RA4	II x I	>10.0, >10.0	1.0
D6 B3 x D22 A9	OL^RB3/1 x OL^RA9	II x I	>10.0, 5.0	1.0
D6 B3 x D22 A14	OL^RB3/1 x OL^RA14	II x II	>10.0, >10.0	>10.0
D6 A4 x D22 A1	OL^RA4/1 x OL^RA1	I x III	>10.0, 2.5	2.5
D6 A4 x D22 A4	OL^RA4/1 x OL^RA4	I x I	>10.0, >10.0	>10.0
D6 A4 x D22 A9	OL^RA4/1 x OL^RA9	I x I	>10.0, 5.0	>10.0
D6 A4 x D22 A14	OL^RA4/1 x OL^RA14	I x II	>10.0, >10.0	1.0
D6 A4 x D22 A20	OL^RA4/1 x OL^RA20	I x I	>10.0, >10.0	>10.0

TABLE 6.12.

Allelism - Recombination Analysis of Diploids from $OL^{R}I\rho^{+}$ x $OL^{R}I\rho^{+}$ Crosses

ASSAY : REPLICA PLATING

Diploids were isolated by mass mating and prototrophic selection (chapter 2). These were plated onto MMGlu, incubated for 3 days at 30°C, and then replicated onto YEPGly medium $\pm$ 2.5 γ/ml oligomycin. After a further incubation at 30°C for 3 days, the plates were scored for the total number of ρ^{+} colonies, as well as for the percentage of colonies resistant or sensitive to oligomycin.

TABLE 6.12.

Cross	Genotypes of OL^R Strains Crossed	Percentage OL^R Colonies	Percentage Mixed Colonies ($OL^R + OL^S$)	Percentage OL^S Colonies	Total ρ^+ Colonies Analysed
D22 A7 D6 2 x D22 A2	OL^RA7 x OL^RA2	100.0	0	0	53
D22 A7 D6 2 x D22 A3	OL^RA7 x OL^RA3	100.0	0	0	506
D22 A7 D6 2 x D22 A5	OL^RA7 x OL^RA5	100.0	0	0	300
D22 A7 D6 2 x D22 A7	OL^RA7 x OL^RA7	100.0	0	0	162
D22 A7 D6 2 x D22 B8	-	-	-	-	-
D22 A7 D6 2 x D22 B9	OL^RA7 x OL^RB9	100.0	0	0	321
D22 A7 D6 2 x D22 B10	OL^RA7 x OL^RB10	100.0	0	0	1084
D22 A7 D6 2 x D22 B16	OL^RA7 x OL^RB16	100.0	0	0	447
D22 A7 D6 11 x D22 A2	OL^RA7 x OL^RA2	99.4	0.6	0	324
D22 A7 D6 11 x D22 B16	OL^RA7 x OL^RB16	100.0	0	0	141
D22 A12 D6 2 x D22 A4	OL^RA12 x OL^RA4	100.0	0	0	655
D22 A12 D6 2 x D22 B9	OL^RA12 x OL^RB9	100.0	0	0	579

TABLE 6.12 (continued)

Cross	Genotypes of OL^R Strains Crossed	Percentage OL^R Colonies	Percentage Mixed Colonies ($OL^R + OL^S$)	Percentage OL^S Colonies	Total ρ^+ Colonies Analysed
D22 A12 D6 2 x D22 B10	OL^RA12 x OL^RB10	100.0	0	0	297
D22 B8 D6 9 x D22 A5	OL^RB8 x OL^RA5	95.0	5.0	0	80
D22 B8 D6 9 x D22 B10	OL^RB8 x OL^RB10	96.0	4.0	0	536
D22 B8 D6 S1 x D22 B9	OL^RB8 x OL^RB9	100.0	0	0	68
D22 B9 D6 C x D22 A2	OL^RB9 x OL^RA2	100.0	0	0	311
D22 B9 D6 C x D22 A3	OL^RB9 x OL^RA3	100.0	0	0	332
D22 B9 D6 C x D22 A5	OL^RB9 x OL^RA5	100.0	0	0	168
D22 B9 D6 C x D22 A7	OL^RB9 x OL^RA7	100.0	0	0	357
D22 B9 D6 C x D22 A20	OL^RB9 x OL^RA20	100.0	0	0	294
D22 B9 D6 C x D22 B8	OL^RB9 x OL^RB8	100.0	0	0	255
D22 B9 D6 C x D22 B10	OL^RB9 x OL^RB10	100.0	0	0	289

TABLE 6.12. (continued)

Cross	Genotypes of OL^R Strains Crossed	Percentage OL^R Colonies	Percentage Mixed Colonies ($OL^R + OL^S$)	Percentage OL^S Colonies	Total ρ^+ Colonies Analysed
D22 B9 D6 C x D22 B15	OL^RB9 x OL^RB15	100.0	0	0	142
D22 B9 D6 C x D22 B16	OL^RB9 x OL^RB16	100.0	0	0	181
D6 A4 x D22 A5	OL^RA4/1 x OL^RA5	100.0	0	0	193
D6 A4 x D22 B8	OL^RA4/1 x OL^RB8	100.0	0	0	140
D6 A9 x D22 A5	OL^RA9/1 x OL^RA5	100.0	0	0	434
D6 A9 x D22 A9	OL^RA9/1 x OL^RA9	100.0	0	0	150
D6 A9 x D22 A20	OL^RA9/1 x OL^RA20	100.0	0	0	219
D6 A9 x D22 B8	OL^RA9/1 x OL^RB8	100.0	0	0	178
D6 A9 x D22 B15	OL^RA9/1 x OL^RB15	100.0	0	0	359

the former group would represent crosses between allelic determinants, and the latter, non-allelic determinants. However, as shown in Table 6.12, diploids from virtually all the crosses were oligomycin resistant when analysed by replica plating. This held true whether the cross was one involving a resistant determinant crossed by itself (e.g. D22 B9 x D22 B9 D6 C) or one involving determinants on independently isolated strains which therefore may or may not be genetically identical. Only two crosses in fact gave other than resistant diploids and these were crosses involving D22 B8 D69 x D22 B10 and D22 B8 D69 x D22 A5. Even in these cases, however, oligomycin sensitive diploid colonies were not observed, the colonies being either oligomycin resistant or apparently mixed.

The results of the three crosses performed involving strain D22 B8 D69 suggest that the oligomycin resistant determinant carried by this strain is identical to that carried by strain D22 B9 whilst different from that carried by strains D22 A5 and D22 B10. However, when crosses of D22 B9 by D22 A5 and D22 B10 were analysed, only resistant diploid progeny were observed, suggesting that these strains all carry identical oligomycin determinants. The replica plating analysis, therefore, currently provides no cohesive evidence for the existence of more than one recombination group. The failure to find coherent evidence of segregation of phenotypic cell types may be explained in several ways :

(i) All the 16 strains carry alleles of the same genetic locus or of closely linked loci. In the latter case an insufficiently large sample of diploids from each cross might have been analysed to detect recombinants. The lowest frequency that could have been detected was 1×10^{-3}

(ii) Recombination does occur but is obscured phenotypically and cannot be detected by replica plating analysis. The finding mentioned above of 'mixed' diploid colonies in certain crosses suggests that this may be the correct explanation.

The results of aliquot plating studies carried out on diploids from crosses of the type $OL^R I \rho^+ \times OL^R I \rho^+$ are shown in Table 6.13. Crosses which appeared to give only resistant diploids on replica analysis were found by aliquot analysis to give mainly sensitive cells, only a minority of resistant cells apparently being present. As the majority of crosses listed in Table 6.13. involved crossing an OL^R determinant by itself (mating type changed) the expectation was that in the

TABLE 6.13.

Quantitative Replica and Aliquot Analysis of Diploids from Class I OL^R x Class I OL^R Crosses

Diploids were isolated by mass mating and prototrophic selection (chapter 2). These were analysed by aliquot plating using MMGly drug plates $\pm$ 2.5 γ/ml oligomycin. Incubation prior to scoring was for 6 days at 30°C. Where two values are given in the table these were obtained in independent crosses. The figures in parentheses refer to the total number of colonies counted on non-selective medium.

TABLE 6.13.

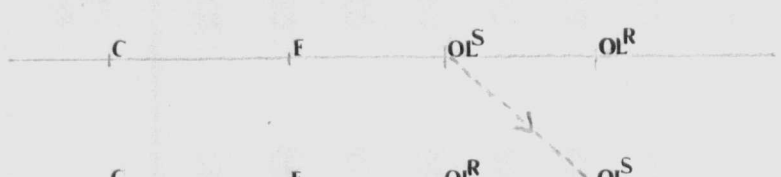
Cross	Percentage Resistant or Mixed Colonies / Total Colonies (Replica Plating)	Percentage Resistant Colonies / Total Colonies (Aliquot Plating)		Colony Size
D22 A7 x D22 A7 D6 2 (OL^RA7 x OL^RA7)	100	2.4	(188)	Homogeneous
D22 B9 x D22 B9 D6 C (OL^RB9 x OL^RB9)	100	73.0, 86.0	(50, 422)	Homogeneous
D22 B8 x D22 B8 D6 S1 (OL^RB8 x OL^RB8)	100	0.8	(50)	Heterogeneous
D22 B8 x D22 B8 D6 9 (OL^RB8 x OL^RB8)	N.T.	0.023	(384)	Heterogeneous
D22 B8 x D22 B8 D6 C1 (OL^RB8 x OL^RB8)	N.T.	0.49	(370)	Heterogeneous
D6 A4 x D22 B8 (OL^RA4/1 x OL^RB8)	100	0.01		Heterogeneous

majority of cases transmission values approaching 100% would be found. In fact, in only one cross was this value approached (D22 B9 x D22 B9 D6C), whilst in other crosses the transmission frequencies measured were as low as 0.023%. Although these transmission frequencies for oligomycin resistance are low, it must be stressed that they were generally at least a thousand-fold greater than those exhibited in crosses of the type $OL^{R}I\rho^{+}$ x $OL^{S}\rho^{+}$. The behaviour in the former crosses is therefore clearly distinguished from that in the latter.

Examination of these results suggests, therefore, firstly that replica plating analysis of this type of mutant is unable to distinguish between crosses involving allelic and non-allelic strains. Secondly, that as many crosses involving a given genetic determinant crossed by itself still show, on aliquot analysis segregation or sensitive cells, either some unknown type of phenotypic interaction is occurring of there must be additional genetic determinants concerned in the conferral of the resistance phenotype. Until the genetics of the class I mutants is better understood, it appears impracticable to obtain any meaningful analysis of the allelism of the isolated strains.

Allelism-recombination analysis of the class II mutants

The results of a recombination analysis of the class II OL^{R} mutants in which D22 OL^{R}II mutants were crossed with D6 OL^{R}II mutants is shown in Table 6.14. In many of these crosses a high proportion of the progeny were oligomycin sensitive, suggesting that recombination between two different loci for oligomycin resistance had occurred. This is schematically illustrated below.



Without a cis-trans test it is difficult to be unequivocal about whether this recombination represents an intergenic or an intragenic event, but in the absence of complicating factors such as gene amplification, the magnitude of the recombination

TABLE 6.14.

Allelism - Recombination Analysis of Diploids from $OL^{R}II\rho^{+} \times OL^{R}II\rho^{+}$ Crosses

ASSAY : REPLICA PLATING

Diploids from crosses of $OL^{R}II\rho^{+} \times OL^{R}II\rho^{+}$ haploids were obtained as described in chapter 2. Suspensions of diploid cells were spread onto MMGlu plates to give single colonies and these were replicated after 3 days incubation at $30^{o}C$ onto YEPGly plates $\pm$ 2.5 γ/ml oligomycin. The replicated plates were scored after a further 3 days incubation at $30^{o}C$.

TABLE 6.14.

Cross	Genotypes of OL^R Strains Crossed	Percentage OL^S Colonies in Diploids	Total ρ^+ Colonies Analysed	Range of OL^S Recombinant Values Recorded
D6 B2 x D22 A13	OL^RB2/1 x OL^RA13	9.2	190	9.0, 8.9
D6 B2 x D22 A14	OL^RB2/1 x OL^RA14	13.5	140	14.6, 11.1, 14.9
D6 B2 x D22 A15	OL^RB2/1 x OL^RA15	5.1	225	4.9, 4.1, 4.3, 5.8
D6 B2 x D22 A16	OL^RB2/1 x OL^RA16	0	157	0, 0, 0
D6 B2 x D22 A18	OL^RB2/1 x OL^RA18	8.8	283	9.7, 5.8, 10.5
D6 B2 x D22 A19	OL^RB2/1 x OL^RA19	<0.3	334	0, 0, 0.5
D6 B2 x D22 A21	OL^RB2/1 x OL^RA21	0	134	0, 0
D6 B2 x D22 B1	OL^RB2/1 x OL^RB1	0	400	0, 0, 0
D6 B2 x D22 C4	OL^RB2/1 x OL^RC4	14.9	161	17.0, 6.0, 20.7

continued .....

TABLE 6.14. (continued)

Cross	Genotypes of OL^R Strains Crossed	Percentage OL^S Colonies in Diploids	Total ρ^+ Colonies Analysed	Range of OL^S Recombinant Values Recorded
D6 B3 x D22 A13	OL^RB3/1 x OL^RA13	-	-	-
D6 B3 x D22 A14	OL^RB3/1 x OL^RA14	19.0	131	24.2, 19.2, 13.2
D6 B3 x D22 A15	OL^RB3/1 x OL^RA15	11.1	27	11.1
D6 B3 x D22 A16	OL^RB3/1 x OL^RA16	0	140	0, 0
D6 B3 x D22 A18	OL^RB3/1 x OL^RA18	10.0	20	10.0
D6 B3 x D22 A19	OL^RB3/1 x OL^RA19	<0.3	296	0, 0.3, 0
D6 B3 x D22 A21	OL^RB3/1 x OL^RA21	0	167	0, 0, 0
D6 B3 x D22 B1	OL^RB3/1 x OL^RB1	0	291	0, 0, 0
D6 B3 x D22 B21	OL^RB3/1 x OL^RB21	4.2	283	4.8, 5.0, 2.5
D6 B3 x D22 C4	OL^RB3/1 x OL^RC4	5.1	337	9.1, 3.5, 2.0

continued .....

TABLE 6.14 (continued)

Cross	Genotypes of OL^R Strains Crossed	Percentage OL^S Colonies in Diploids	Total ρ^+ Colonies Analysed	Range of OL^S Recombinant Values Recorded
D6A1 x D22A13	OL^RA1/1 x OL^RA13	15.5	283	16.0, 14.2, 16.0
D6A1 x D22A14	OL^RA1/1 x OL^RA14	15.1	172	11.5, 11.1, 19.4
D6A1 x D22A15	OL^RA1/1 x OL^RA15	6.75	829	2.5, 8.2, 2.2, 8.5
D6A1 x D22A16	OL^RA1/1 x OL^RA16	0	138	0
D6A1 x D22A18	OL^RA1/1 x OL^RA18	4.2	266	2.4, 5.3, 5.0
D6A1 x D22A19	OL^RA1/1 x OL^RA19	-	-	-
D6A1 x D22A21	OL^RA1/1 x OL^RA21	0.38	804	0, 0.68, 0.3
D6A1 x D22B1	OL^RA1/1 x OL^RB1	0	341	0
D6A1 x D22C4	OL^RA1/1 x OL^RC4	20.8	67	29.0, 10.0

continued .....

TABLE 6.14. (continued)

Cross	Genotypes of OL^R Strains Crossed	Percentage OL^S Colonies in Diploids	Total ρ^+ Colonies Analysed	Range of OL^S Recombinant Values Recorded
D6 A5 x D22 A13	OL^RA5/1 x OL^RA13	13.7	182	12.9, 18.2, 9.6
D6 A5 x D22 A14	OL^RA5/1 x OL^RA14	9.7	299	10.3, 10.1, 8.6
D6 A5 x D22 A15	OL^RA5/1 x OL^RA15	-	-	-
D6 A5 x D22 A16	OL^RA5/1 x OL^RA16	-	-	-
D6 A5 x D22 A18	OL^RA5/1 x OL^RA18	3.7	161	3.8, 7.1, 0
D6 A5 x D22 A21	OL^RA5/1 x OL^RA21	0	208	0, 0, 0
D6 A5 x D22 B1	OL^RA5/1 x OL^RB1	0	355	0, 0, 0
D6 A5 x D22 C4	OL^RA5/1 x OL^RC4	10.4	211	11.5, 10.3, 9.2

event leads one to suggest the former. The presence of oligomycin sensitive diploids was not due to a carry-over of oligomycin sensitive colonies from impure haploid strains, as the precultures used for the mating were grown on selective medium containing oligomycin.

These preliminary experiments (Table 6.14) show that there are certainly two recombination groups present. The spread of recombination values observed was such, however, that it remained a possibility that there were three or even four loci determining cytoplasmic oligomycin resistance. In order to investigate this further, a strain carrying determinant $OL^{R}A15$ (the same gene as is carried by strain D22A15) but with altered mating type, was constructed and has been mated to all the D22 derived strains which showed recombination with D6B3, D6A1 and D6B2. As these strains, to wit D22A13, D22A14, D22A15, D22A18 and D22C4 did not show any recombination with this strain carrying gene $OL^{R}A15$, two cytoplasmic loci and two alone appear to be responsible for conferring oligomycin resistance in the strains isolated. The strains and their allocation in the two recombination groups are shown in Table 6.15.

It is interesting to note that J. R. Turner (J.R.Turner, private communication) has obtained biochemical data which suggests that the shape of the mitochondrial ATPase/oligomycin inhibition curves may be different for mutants in the different allelic groups. Experiments have been carried out with Professor Slonimski's group, crossing various strains from both allelic groups 'R' and 'S' by two of their strains IL778-3D and IL836-4B, which both carry the oligomycin resistant determinant $O^{R}1$. The results of this analysis (Table 6.16) show that determinants $OL^{R}A16$, $OL^{R}A21$ and $OL^{R}A1/1$ are allelic to $O^{R}1$ whilst $OL^{R}A13$ and $OL^{R}A15$ are non-allelic. The percentage of oligomycin sensitive recombinants observed did not differ significantly between diploid progeny from D22A15 x IL836-4B and D22A15 x IL778-3D. As IL836-4B is an ω^{+} strain and IL778-3D an ω^{-} strain, there is therefore no apparent discrimination between the recombination value observed for these loci in homosexual and heterosexual crosses.

Further Genetic Analysis - Class II Mutants

Mitochondrial 'sexing' of parental strains D22 and D6

Table 6.17. shows the results obtained in two-point crosses between the standard ω^{+} and ω^{-} strains defined in the laboratory of Professor Slonimski, carrying

TABLE 6.15.

Summary of Table 6.13. Allelism-Recombination Data

Recombination Group 'R'		Recombination Group 'S'
D6A1)		
)		
D6A5)		D22A13
)	Testers	
D6B2)		D22A14
)		
D6B3)		D22A15
D22A16		D22A18
D22A19		D22B21
D22A21		D22C4
D22B1		

TABLE 6.16.

Recombination - Extended Allelism Analysis

ASSAY : REPLICA PLATING

Crosses were made using the procedure outlined in chapter 2. The genotype of the diploid colonies was assessed by replica plating analysis.

TABLE 6.16.

Cross	OL^R Genotypes	Percentage OL^S Recombinants	Total ρ^+ Colonies Analysed
D22 A13 x IL 778-2D	$\rho^+ OL^R A13 \times \rho^- O^R 1$	11.9	428
D22 A13 x IL 836-4B	$\rho^+ OL^R A13 \times \rho^+ O^R 1$	12.8	1078
D22 A15 x IL 778-2D	$\rho^+ OL^R A15 \times \rho^- O^R 1$	14.1	514
D22 A15 x IL 836-4B	$\rho^+ OL^R A15 \times \rho^+ O^R 1$	11.4	693
D22 A16 x IL 778-2D	$\rho^+ OL^R A16 \times \rho^- O^R 1$	0	688
D22 A16 x IL 836-4B	$\rho^+ OL^R A16 \times \rho^+ O^R 1$	0	803
D22 A21 x IL 778-2D	$\rho^+ OL^R A21 \times \rho^- O^R 1$	0	616
D22 A21 x IL 836-4B	$\rho^+ OL^R A21 \times \rho^+ O^R 1$	0	1155
D6 A1 x IL 778-1A	$\rho^+ OL^R A1/1 \times \rho^- O^R 1$	0	127
D6 A1 x IL 781-1A	$\rho^+ OL^R A1/1 \times \rho^+ O^R 1$	0	151

TABLE 6.17.

Two Point 'Mitochondrial' Crosses - 'Mitochondrial Sexing'

ASSAY : REPLICA PLATING

Crosses were made using the procedure outlined in chapter 2. The genotype of the diploid colonies was assessed by replica plating analysis.

TABLE 6.17.

Cross	Percentage Transmission		Percentage of Total ρ^+ Colonies				Total Colony Count ρ^+
	C^R	E^R	C^SE^S	C^SE^R	C^RE^S	C^RE^R	
D22 x IL8-8C ($\rho^+C^SE^S \times \omega^+\rho^+C^RE^R$)	65.3	65.7	28.6	3.5	5.3	61.4	283
D22 x IL126-3A ($\rho^+C^SE^S \times \omega^-\rho^+C^RE^R$)	0.40	31.6	68.1	31.1	0	2.4	405
D6 x IL8-8D ($\rho^+C^SE^S \times \omega^+\rho^+C^RE^R$)	87.7	91.9	5.7	6.6	2.4	85.3	211
D6 x IL126-1C ($\rho^+C^SE^S \times \omega^-\rho^+C^RE^R$)	8.9	73.0	26.9	64.0	0	8.9	156

the cytoplasmically inherited resistance alleles C^R_{321} E^R_{514} and C^R_{321} E^R_{221} (Coen et al., 1970; Bolotin et al., 1971) and D6 and D22 respectively - both of which are $C^S E^S$. The E^R_{514} and E^R_{221} alleles appear to be identical (Slonimski, private communication) and the strains are also essentially isochromosomal,* differing apparently only in their 'mitochondrial sex.'

The results of the crosses of D6 and D22 with the ω^- strains IL 126-1C and IL 126-3A show the polarity of recombination typical of a heterosexual cross, that is one involving $\omega^+ \times \omega^-$ (Coen et al., 1970; Bolotin et al., 1971), with more $C^S E^R$ than $C^R E^S$ recombinants. This suggests that both D6 and D22 must carry the ω^+ allele. This is confirmed by the crosses with the ω^+ $C^R E^R$ strains IL 8-8C and IL 8-8D where the relative proportions of $C^S E^R$: $C^R E^S$ show a lack of polarity - behaviour characteristic of homosexual crosses. This means that both D6 and D22 are ω^+ as this is the allele carried by strains IL 8-8D and IL 8-8C.

Whilst this behaviour is clear cut, D22 behaving identically to all the ω^+ strains examined by Slonimski (private communication), D6 shows certain atypical characteristics. For instance, in the heterosexual cross D6 x IL 126-3A, the frequency of recombination between the C^R and E^R loci is much greater than that observed in any other heterosexual crosses involving ρ^+ strains (Slonimski, private communication). This suggests that there is a 'scale expansion' or greatly increased recombination frequency, at least in the region of the genome between the C^R and E^R determinants, and that this behaviour is conditioned by an allele that D6 carries. Similarly, the behaviour of D6 in the homosexual cross with IL 8-8D shows a greatly increased preponderance of the double resistant diploid $C^R E^R$ compared with other crosses involving IL 8-8D (Slonimski, private communication). The precise meaning of these observations is not clear, but it appears that the crosses of D22 OL^R or D22 E^R x D6 OL^S E^S, and vice versa, are basically homosexual mitochondrial crosses. The various peculiarities noted, especially the high preponderance of the IL 8-8D genotype $C^R E^R$ amongst diploids of the homosexual cross IL 8-8D x D6 would, if repeated in crosses of D6 x D22 explain the notable asymmetry of both oligomycin and erythromycin resistance transmission in these crosses. This asymmetry of transmission, as already noted, depends on whether the allele for resistance is carried by strain D6 or D22 (Table 6.2).

* Highly inbred

Recombination studies between the C^R, E^R and OL^R determinants

Table 6.18. shows preliminary data obtained by crossing the cytoplasmically inherited erythromycin resistant strain D22 Er310 by three of the D6 OL^RII mutants. In agreement with Stuart (Stuart, 1970) recombination was found to occur between these two loci. The total percentage of recombinants (i.e. classes $E^R OL^R$ + $E^S OL^S$) was, however, much lower than that found in similar homosexual crosses by Slonimski et al. (private communication). This behaviour is probably due in the most part to the anomalous behaviour of D6 and derived strains which, as already noted, causes markedly asymmetric transmission of the two parental genotypes in homosexual crosses.

Attempts to map the two non-allelic groups of class II oligomycin resistant mutants have been undertaken. This work has been carried out in collaboration with Professor Slonimski and his research group. The results show clear differences in behaviour between mutants belonging to the different allelism groups 'R' and 'S' whilst mutants within the same group show similar behaviour (Table 6.19). In heterosexual crosses, polarity between E - OL is much weaker in crosses involving D22 A16 and D22 A21 than it is in those involving D22 A15 and D22 A13. The polarity in the E - OL region of the latter two strains, however, appears similar to that shown by strains carrying the gene O^R1 despite this determinant being non-allelic with OL^RA15 and OL^RA13, the determinants carried by D22 A15 and D22 A13. The reason for this discrepancy is not known but there is some indication that the polarity between C - E is weaker in all crosses of the ω^+ D22 strains by ω^- strains such as IL 126-3A in comparison to the results obtained with the ω^+ strains of Slonimski (Coen et al., 1970; Bolotin et al., 1971).

The results from the three-point mapping with the heterosexual crosses therefore, suggest that $O^R1 = OL^R A15 = OL^R A13 \neq OL^R A16 = OL^R A21$, a result concerning O^R1 in disagreement with the allelism data. A similar discrepancy in recombination frequencies between the E and OL genes is also apparent on analysis of the recombination data from the homosexual crosses with strain IL 8-8C (Table 6.19). The reason for these results is not understood but obviously render the localisation of the oligomycin determinants of allelism groups 'R' and 'S' by three-point mapping impossible.

Further experiments have, in fact, confirmed the correctness of the allelism

TABLE 6.18.

Strains were mass mated (chapter 2) and the resulting diploids spread on MMGlu, grown for two days, and then replicated onto YEPGly + erythromycin (4 mg/ml) + oligomycin (2.5 γ/ml); YEPGly + erythromycin (4 mg/ml); YEPGly + oligomycin (2.5 γ/ml) and YEPGly plates in that order. Plates were incubated for 2 - 3 days at 30°C before scoring.

TABLE 6.18.

Cross	Percentage Transmission E^R	Percentage Transmission OL^R	Percentage $E^R OL^R$	Percentage $E^R OL^S$	Percentage $E^S OL^S$	Percentage $E^S OL^R$	Total Colonies
D6 B3 x D22 Er 310 ($w^+ Er^S OL^R$ B3/1 x $w^+ Er_3^R OL^S$)	91.0	7.5	0	91.0	1.5	7.5	534
D6 B1 x D22 Er 310 ($w^+ Er^S OL^R$ B1/1 x $w^+ Er_3^R OL^S$)	89.3	18.2	7.2	81.1	0.7	11.0	610
D6 A1 x D22 Er 310 ($w^+ Er^S OL^R$ A1/1 x $w^+ Er_3^R OL^S$)	84.1	20.0	5.7	78.4	1.6	14.3	385

TABLE 6.19.

Three Point Crosses

Diploids were obtained by mass mating and spread to obtain single colonies on MMGlu plates. After 2 - 3 days incubation at 28°C these colonies were replicated onto YEPGly + CAP + Er, YEPGly + CAP + OL, YEPGly + CAP, YEPGly + ER, YEPGly + OL and YEPGly plates. The concentration of CAP in the media was 4.0 mg/ml, of ER 5 mg/ml and of OL 2.5 γ/ml. After replicating plates were incubated at 28°C for 2 - 3 days in the case of oligomycin, erythromycin and YEPGly plates and for 5 days in the case of all the other types of media.

TABLE 6.19.

Cross	$C^RE^SOL^S$	$C^RE^SOL^R$	$C^SE^ROL^S$	$C^SE^ROL^R$	$C^SE^SOL^S$	$C^SE^SOL^R$	$C^RE^ROL^S$	$C^RE^ROL^R$	Σcol
* D22A16 x IL126-3A ($_{\infty}{}^{+}C^SE^SOL^R$ A16 x $_{\infty}{}^{-}C^RE^ROL^S$)	0	0	10.1	7.5	11.7	70.7	0	0	573
* D22A16 x IL8-8C ($_{\infty}{}^{+}C^SE^SOL^R$ A16 x $_{\infty}{}^{+}C^RE^ROL^S$)	1.7	1.7	1.4	2.2	4.5	32.0	48.0	8.4	356
* D22A21 x IL126-3A ($_{\infty}{}^{+}C^SE^SOL^R$ A21 x $_{\infty}{}^{-}C^RE^ROL^S$)	0.2	0	21.6	13.0	13.8	50.0	1.0	0.5	616
* D22A21 x IL8-8C ($_{\infty}{}^{+}C^SE^SOL^R$ A21 x $_{\infty}{}^{+}C^RE^ROL^S$)	3.0	1.5	0.4	0.7	5.2	28.5	51.7	9.0	267
** D22A15 x IL126-3A ($_{\infty}{}^{+}C^SE^SOL^R$ A15 x $_{\infty}{}^{-}C^RE^ROL^S$)	0	0	10.0	3.3	21.9	62.9	1.4	0.5	210
** D22A15 x IL8-8C ($_{\infty}{}^{+}C^SE^SOL^R$ A15 x $_{\infty}{}^{+}C^RE^ROL^S$)	1.2	3.0	2.4	2.4	8.9	23.7	52.1	6.5	169

continued .....

TABLE 6.19. (continued)

Cross	$C^{R}E^{S}OL^{S}$	$C^{R}E^{S}OL^{R}$	$C^{S}E^{R}OL^{S}$	$C^{S}E^{R}OL^{R}$	$C^{S}E^{S}OL^{S}$	$C^{S}E^{S}OL^{R}$	$C^{R}E^{R}OL^{S}$	$C^{R}E^{R}OL^{R}$	Σcol
** D22A13 x IL126-3A ($_{\infty}{}^{+}C^{S}E^{S}OL^{R}$ A13 x $_{\infty}{}^{-}C^{R}E^{R}OL^{S}$)	0.3	0	23.4	4.8	20.4	47.6	2.5	1.0	393
** D22A13 x IL8-8C ($_{\infty}{}^{+}C^{S}E^{S}OL^{R}$ A13 x $_{\infty}{}^{+}C^{R}E^{R}OL^{S}$)	3.1	3.1	1.6	1.6	7.1	19.7	59.9	3.9	254

	Polarity	** D22A15	** D22A13	* D22A21	* D22A16
C - E	$\frac{C^{S}E^{R}}{C^{R}E^{S}}$	$\frac{13.3}{0} = \infty$	$\frac{28.2}{0.3} = 93.9$	$\frac{34.6}{0.2} = 173.0$	$\frac{17.6}{0} = \infty$
E - OL	$\frac{E^{S}OL^{S}}{E^{R}OL^{R}}$	$\frac{21.9}{3.8} = 5.9$	$\frac{20.7}{5.8} = 3.6$	$\frac{14.0}{13.5} = 1.0$	$\frac{11.7}{7.5} = 1.6$

Analysis of Heterosexual Crosses

* Recombination Group 'R'

** Recombination Group 'S'

data, whilst the use of petites with defined deletions of mDNA and u.v. irradiation mating experiments have allowed unambiguous localisation of the two groups of determinants in relationship to the C^R, E^R and S^R determinants (Bolotin et al., 1971) (data to be published).

Further genetic analysis of the class I mutants

In view of the apparent anomalous behaviour of the class I mutants, genetic experiments were undertaken to clarify :

(a) whether the oligomycin and cross resistances shown by these mutants were due to a single gene showing pleiotropic effects or due to multiple gene mutations

(b) the nature of the genetic determinant causing this resistance, bearing in mind the apparent non-mendelian behaviour class I mutants show in crosses yet mendelian behaviour on tetrad analysis, and the failure to find correlation between the resistance transmission frequencies as measured by aliquot and replica plating (Table 6.4).

The uni- or multi-genic nature of the determinant responsible for resistance in the class I mutants was investigated by crossing the resistant haploids to a sensitive tester (ρ^+) and recovering haploids from these diploids by sporulation. These recovered haploids were then examined to see whether any possessed oligomycin resistance without the associated cross resistances or vice versa. Failure to find alteration in this relationship would, provided a large enough sample were analysed, provide strong presumptive evidence that only a single gene or tightly linked genes were responsible for all the resistances. On the other hand, loss of this relationship does not necessarily imply that more than one determinant is directly concerned in this resistance pattern as in an integrated membrane system, segregation of secondary elements might, by interaction, cause the loss of the cross resistances. Apirion and coworkers (Apirion et al., 1969) for instance, have shown that neomycin resistance in E.coli can be suppressed in strains which carry a determinant for spectinomycin resistance. The opposite also holds true. Both these mutations confer their respective resistances by altering ribosomal proteins. The suppression of the resistances in the double mutant is apparently due to a membrane interaction at the level of the ribosome (Apirion et al., 1969). Table 6.20 shows that none of the oligomycin resistant haploids recovered have lost cross resistance to all the antibiotics to which the original haploid was resistant

TABLE 6.20.

Cross Resistance Analysis of Haploids Obtained from $OL^{R}I\rho^{+}$ x $OL^{S}\rho^{+}$ Backcrosses

D22 derived class I mutants (a $OL^{R}ad_{2}\rho^{+}$) were taken and mated to D6 (α OL^{S} arg. met. ρ^{+}) and diploids isolated and sporulated as described in chapter 2. Haploids were re-isolated from these sporulated strains by random re-isolation of spores (chapter 2). Following plating out of these strains on YEPG, they were examined for their mating type and auxotrophic requirements. Strains which were of a mating type and auxotrophic for arginine and methionine, but not adenine, were tested for their oligomycin and cross resistances by dropping out onto YEPGly plates $\pm$ drug. Incubation was for 3 days at 30°C. The drug concentrations used were : oligomycin 5.0 γ/ml, erythromycin 0.75 and 1 mg/ml, spiramycin 1.0 and 1.5 mg/ml, chloramphenicol 1.0 and 2.0 mg/ml.

TABLE 6.20.

Strain	OL^R Genotype	Oligomycin Resistance	Erythromycin Resistance	Spiramycin Resistance	Chloramphenicol Resistance
D22 A7 D6 1	OL^R A7	-	-	-	-
D22 A7 D6 2	OL^R A7	+	+	+	+
D22 A7 D6 11	OL^R A7	+	+	+	+
D22 B8 D6 1	OL^R B8	+	+	+	+
D22 B8 D6 9	OL^R B8	+	+	+	+
D22 B8 D6 12	OL^R B8	+	-	-	+
D22 B8 D6 C1	OL^R B8	+	+	+	+
D22 B8 D6 J1	OL^R B8	+	+	+	+
D22 B8 D6 N1	OL^R B8	+	+	+	+
D22 B8 D6 S1	OL^R B8	+	-	-	+
D22 B8 D6 2	OL^R B8	-	-	-	-
D22 B8 D6 9(1)	OL^R B8	-	-	-	-
D22 B9 D6 C	OL^R B9	+	-	+	+
D22 A20 D6 H1	OL^R A20	+	+	+	+
D22 A20 D6 N	OL^R A20	+	+	+	+
D22 A3 D6 4	OL^R A3	-	-	-	-
D22 A12 D6 3	OL^R A12	-	-	-	-
D22 B5 D6 A10	OL^R B5	-	-	-	-
D22 B10 D6 1	OL^R B10	-	-	-	-
D22	-	-	-	-	-
D6	-	-	-	-	-

simultaneously. All the recovered oligomycin resistant strains have retained their resistance to chloramphenicol and the majority of the recovered resistant haploids have also retained their erythromycin and spiramycin resistance (Table 6.20). Similarly, the oligomycin sensitive haploids recovered from these $OL^{R}I\rho^{+}$ x $OL^{S}\rho^{+}$ crosses have, in all cases, failed to show associated cross resistance to erythromycin, spiramycin and chloramphenicol.

These results, therefore, suggest that one gene, and one alone, is responsible for the primary resistance to oligomycin and the associated cross resistance pattern. For instance, the failure to lose all three of the cross resistances simultaneously and the loss of erythromycin resistance without spiramycin or chloramphenicol resistance (strain D22 B9 D6 C) makes impossible a multigenic explanation of these results unless one is prepared to postulate that in these mutants 8 - 10 'genes' have been mutated during the original selection of oligomycin resistant mutants. That is to say, an individual 'gene' for each drug or class of drugs to which cross resistance has been developed. For this reason the occasional loss of cross resistance to erythromycin or mikamycin in the recovered haploids is not interpreted as being due to recombination between a gene for oligomycin resistance and separate determinants for cross resistance. As the backcross performed during this analysis involved the mating of two non isochromosomal strains, it appears more probable that the slight changes in the cross resistance pattern are due to reassortment of other genetic determinants affecting membrane function.

The very low transmission rates observed in diploids from $OL^{R}I\rho^{+}$ x $OL^{S}\rho^{+}$ crosses, when analysed by aliquot plating, suggest that the diploid is unstable phenotypically and possibly genotypically. An explanation of the phenomena observed in the diploids, similar to the catabolite repression by glucose found to influence the petite phenotype in the gi mutant (Horn and Wilkie, 1966), does not seem, however, tenable. In order to investigate this problem further, synchronised zygotes of D22 B9 x D6 ($OL^{R}I\rho^{+}$ x $OL^{S}\rho^{+}$) were obtained following the procedure outlined in chapter 2. These zygotes were grown in MMGlu and samples assayed for total cell number and number of oligomycin resistant cells after various periods of growth. The results are shown in Figure 6.2. After a lag period, the percentage of oligomycin resistant diploids was found to fall continuously with time. This was concommitant with an increase in the total cell number. With the exception of that

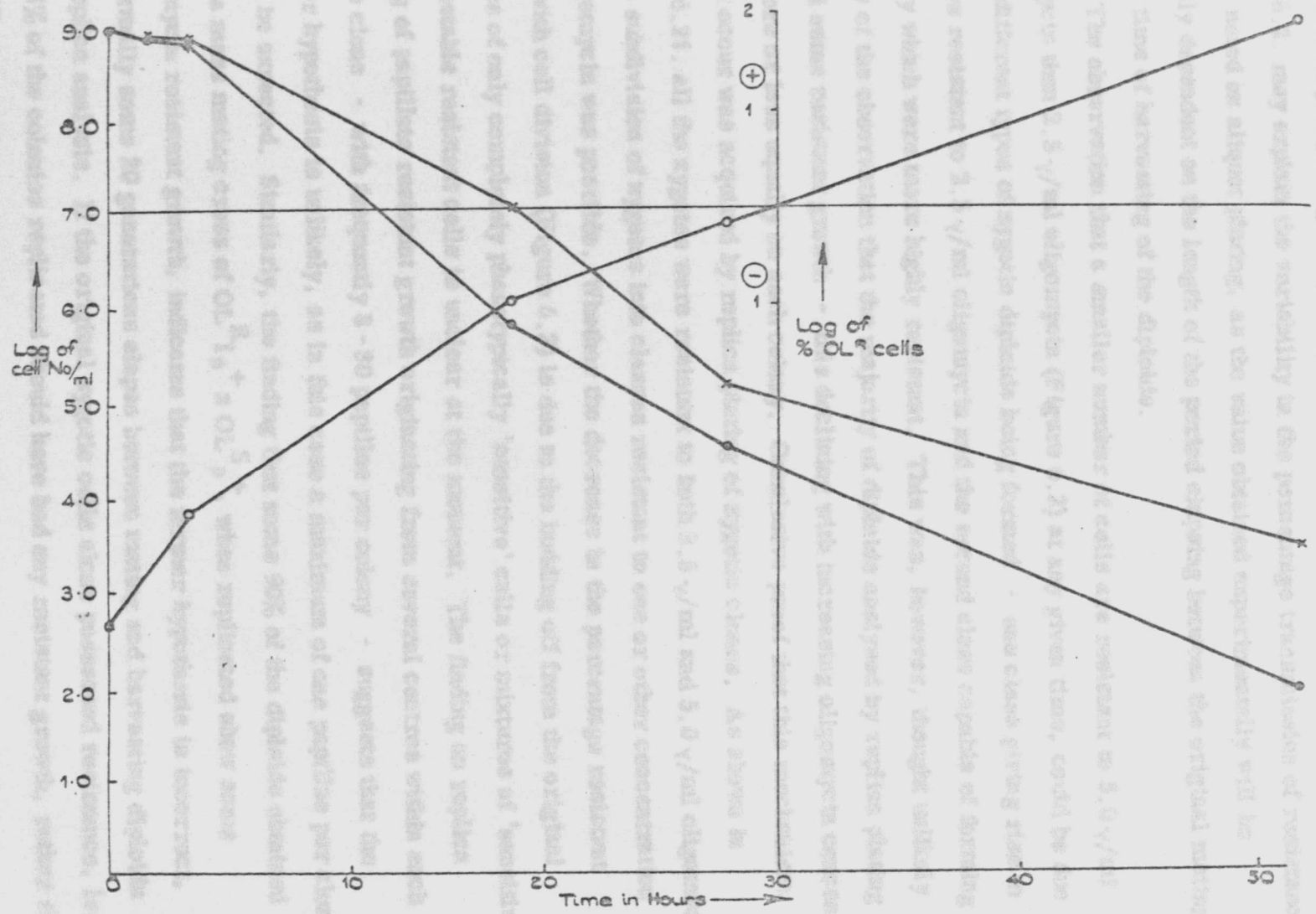
LEGEND TO FIGURE 6.2.

Synchronised zygotes were obtained using the procedure outline in chapter 2. A 1% inoculum of the resulting zygotes was added to 10 ml of MMGlu and this culture was grown at 30°C on a shaking incubator. Samples were taken after 0, 4.5, 19, 28 and 52 hours growth. At each sampling time both cell growth and the percentage of diploids resistant to 2.5 and 5.0 γ/ml oligomycin were estimated by plating onto MMGlu and MMGly ± oligomycin. As the diploids at the 0 and 4 hour samplings failed to grow on MMGly, determinations of cell growth at these times were made by plating onto MMPDM plates. Oligomycin resistant colonies were identified by plating onto MMPDM plates + oligomycin and the resistant colonies noted by using a tetrazolium overlay - the oligomycin resistant colonies were both larger than the oligomycin sensitives and tetrazolium positive. Incubation of the plated cells was at 30°C and scoring carried out after 6 - 8 days.

o % viable cells

x % cells resistant to 2.5 γ/ml oligomycin

• % cells resistant to 5.0 γ/ml oligomycin


9·0
8·0
7·0
6·0
5·0
4·0
3·0
2·0
1·0
Log of cell No/ml
2
+
1
−
1
Log of % OL^{R} cells
0
10
20
30
40
50
Time in Hours

period immediately following the inoculation of the zygotes into the growth media, a higher proportion of the cells were always resistant to 2.5 γ/ml oligomycin than were resistant to 5.0 γ/ml. This finding reinforces the earlier data found on replica and aliquot plating analyses (Tables 6.2. and 6.3). The results in Figure 6.2. may explain the variability in the percentage transmission of resistance already noted on aliquot plating, as the value obtained experimentally will be critically dependent on the length of the period elapsing between the original mating and the time of harvesting of the diploids.

The observation that a smaller number of cells are resistant to 5.0 γ/ml oligomycin than 2.5 γ/ml oligomycin (Figure 6.2) at any given time, could be due to two different types of zygotic diploids being formed - one class giving rise to colonies resistant to 2.5 γ/ml oligomycin and the second class capable of forming progeny which were more highly resistant. This was, however, thought unlikely in view of the observation that the majority of diploids analysed by replica plating showed some resistant growth - this declining with increasing oligomycin concentration more or less equally on each colony. Conclusive proof that this mechanism did not occur was acquired by replica plating of zygotic clones. As shown in Table 6.21. all the zygotes were resistant to both 2.5 γ/ml and 5.0 γ/ml oligomycin and no subdivision of zygotes into classes resistant to one or other concentration of oligomycin was possible. Whether the decrease in the percentage resistant cells with cell division (Figure 6.2) is due to the budding off from the original zygotes of only completely phenotypically 'sensitive' cells or mixtures of 'sensitive' and unstable resistant cells is unclear at the moment. The finding on replica plating of papillate resistant growth originating from several centres within each zygote clone - with frequently 8 -30 papillae per colony - suggests that the former hypothesis is unlikely, as in this case a maximum of one papillae per clone would be expected. Similarly, the finding that some 90% of the diploids obtained from a mass mating cross of $OL^{R}I\rho^{+}$ x $OL^{S}\rho^{+}$, when replicated show some oligomycin resistant growth, indicates that the former hypothesis is incorrect, as normally some 20 generations elapse between mating and harvesting diploids for replica analysis. If the original zygotic cells alone possessed resistance, less than 1% of the colonies replicated should have had any resistant growth, rather than the 90% observed.

TABLE 6.21.

Quantitative Clonal Replica Analysis of Zygotic Diploids from a D22 B9 x D6 Cross

Diploid zygotes from a D22 B9 x D6 cross were obtained using essentially the same procedure as used to obtain synchronous zygotes (chapter 2). Zygotes were immediately plated onto MMGlu and incubated at 30°C for 3 days when they were replicated onto MMGly, MMGly + 2.5 γ/ml oligomycin and MMGly + 5.0 γ/ml oligomycin. Colonies were scored for growth after 3 and 5 days. 'Resistant' clones are those showing confluent growth in the presence of oligomycin; 'mixed' clones show marked papillate growth.

TABLE 6.21.

Level of Oligomycin Used in Test	Total No. of Clones	No. of Resistant Clones	No. of Mixed Clones	No. of Sensitive Clones	% Resistant Clones / Total Clones
SCORED AFTER 3 DAYS					
2.5 γ/ml	556	556	0	0	100
5.0 γ/ml	556	357	7	192	65
SCORED AFTER 5 DAYS					
2.5 γ/ml	556	556	0	0	100
5.0 γ/ml	556	551	5	0	99

As shown in Table 6.4. there is a complete lack of correlation between the results of aliquot and replica plating analysis of diploids from $OL^{R}1\ \rho^{+} \times OL^{S}\ \rho^{+}$ crosses. These two analytical procedures differ in two major respects. Firstly, the density of cells plated in contact with each other on drug plates is much greater on replica than on aliquot plating, and secondly, there is an additional period of growth inherent to the replica plating procedure prior to exposure of the diploids to oligomycin. In order to test the effects of cell density on the resistance transmission, aliquot plating experiments were carried out using diploids from crosses of D22 B9 x D6 and D22 A2 x D6, changing the density at which the cells were plated and scoring the number of resistant cells arising as a function of cell density. The results are shown in Table 6.22. When the resistance transmission frequency is normalised to a standard cell density, it can be seen that the cell density used in the plating experiments is without significant effect. This result strongly argues against any intercellular genetic exchange being involved in the discrepancy between the aliquot and replica plating results.

The intervention of a growth period would normally be expected to affect all the cells equally, if this growth period does not involve segregation of genetic determinants. As it does not apparently, the phenotype itself may be unstable in the diploid and the OL^{R} gene itself capable of being alternatively active or inactive. Although at the concentrations of oligomycin used routinely in these experiments no anomalous behaviour was seen in diploids from D22 $OL^{S}\ \rho^{+}$ x D6 $OL^{S}\ \rho^{+}$ crosses, experiments were undertaken to see the effects, if any, of much lower oligomycin concentrations on these diploids. The results show that providing the concentration of oligomycin used in the media was no greater than 1.0 γ/ml, diploids from this cross showed papillate growth markedly similar to that expressed by diploids from $OL^{R}1$ x OL^{S} crosses at much higher oligomycin concentrations. D6 is known to have a slightly higher oligomycin resistance than D22, 1.0 : 0.5 γ/ml and it appears that this is sufficient differential for the demonstration of similar behaviour between the two wild type alleles to that shown in $OL^{R}1$ x OL^{S} crosses. It can be concluded, therefore, that although this anomalous behaviour may be either characteristic of the particular cistron affected by the $OL^{R}1$ mutation, or of the interaction of this cistron with the rest of the mitochondrial and nuclear genome of the diploid, it is not a characteristic caused by the alteration in the base sequence of this cistron

TABLE 6.22.

Effect of Cell Density on Transmission of Resistance by Diploids from $OL^{R}1\rho^{+}$ x $OL^{S}\rho^{+}$ Cross

Diploids were obtained by prototrophic selection as described in chapter 2. Serial dilutions of suspensions of these diploids were then plated out onto MMGly plates + 2.5 γ/ml oligomycin and incubated at 30°C for 6 - 8 days when cell numbers at each dilution were estimated.

Results in colume 3 = column 2 x dilution factor differences between that dilution and dilution 'N'.

TABLE 6.22.

Cross	Dilution of the Cell Suspension Spread	No. of Resistant Cells/ml at Cell Dilution Used	Resistant Cells/ml of Suspension Spread, Normalised to Dilution Value 'N'	% (No. of Resistant Cells/ml Normalised to Dilution 'N' / Resistant Cells/ml Observed at Dilution 'N')
D22 B9 x D6 $OL^{R}I_{\rho}+$ x $OL^{S}{}_{\rho}+$	'N'	2480.0	2480.0	100
	'N2'	1240.0	2480.0	100
	'N5'	555.0	2775.0	> 100
	'N10'	257.0	2570.0	> 100
	'N20'	120.0	2400.0	~ 100
	'N50'	47.5	2375.0	~ 100
	'N100'	21.0	2100.0	~ 100
D22 A2 x D6 $OL^{R}I_{\rho}+$ x $OL^{S}{}_{\rho}+$	'N'	4800.0	4800.0	100
	'N2'	2400.0	4800.0	100
	'N5'	960.0	4800.0	100
	'N10'	505.5	5055.0	> 100

during mutation to the $OL^R I$ type allele. This just makes the effect visible at higher oligomycin concentrations. Attempts to demonstrate an influence of the rest of the cell genome on this behaviour have so far only used $OL^R I$ resistant spores which were isolated from diploids of D22 $OL^R I$ x D6 crosses. The isolated spores as yet tested all show similar oligomycin resistance transmission rates when crossed to the sensitive testers D6 and D22 (tested by aliquot plating), so such genetic re-assortment as has taken place is without noticeable effect. Further analysis using spores isolated from crosses involving completely different sensitive tester strains are now in progress.

Attempts have been made to combine the class I type of oligomycin resistance with cytoplasmically inherited erythromycin resistance in one and the same cell, by isolating double mutants. The procedure used was simply to plate out cells of D22 B9 at high density onto YEPGly plates containing 4.0 mg/ml erythromycin. The colonies that grew were purified and tested for resistance on oligomycin, erythromycin and oligomycin + erythromycin drug plates. Of the 30 mutants isolated, however, only one has proved to be a double mutant, and this mutant is unable to cross with any of the haploid tester strains in our possession. All the other colonies showed both resistances when tested singly but failed to show resistance when tested on the double drug plates. They therefore presumably either possess a mixed population of mitochondria or are, for some reason, unable to express the two resistances simultaneously. The latter is, however, rather difficult to visualise as the class I OL^R determinant is apparently nuclear whilst erythromycin resistant mutants isolated by this method are generally cytoplasmically inherited (unpublished results). An alternative explanation is that oligomycin and erythromycin interact together when present in the same media to give spurious results. Such a possibility has been suggested independently by Slonimski (private communication). This, under our conditions, seems unlikely as recombination studies between $OL^R I$ strains and D22 Er 310, a ctyoplasmic erythromycin resistant strain, have shown that the double resistant diploids $OL^R I E^R$ can be obtained and differentiated from the complementing strain having both $OL^R I$ and E^R resistances which are not, however, expressed together. The results are shown below. It should be emphasised that these results were obtained by taking the diploids from the cross and plating onto media containing oligomycin, and replicating these oligomycin resistant colonies onto the other drug

media used. Attempts at replicating the diploid colonies from non-selective media onto all the drug media were not carried out as the definition of which colonies are oligomycin resistant is impossible under these conditions (see rest of chapter).

Cross	Genotype	Total no. of colonies analysed	% $E^{R}OL^{R}$	% $E^{S}OL^{R}$	% $E^{R}+OL^{R}$
D22 A7 D6 $\frac{11}{310}$ x D22 Er	OL^{R}A7 x Er^{R}3	37	66.0	31.5	2.5
D22 B9 D6 $\frac{C}{310}$ x D22 Er	OL^{R}B9 x Er^{R}3	22	9.0	73.0	18.0
D22 A7 D6 $\frac{2}{310}$ x D22 Er	OL^{R}A7 x Er^{R}3	5	60.0	20.0	20.0

It does appear from this data therefore, that diploids at least, may possess and express their class I OL^{R} and E^{R} resistances simultaneously.

Discussion

The results presented in this chapter establish genetically the validity of the separation of the oligomycin resistant mutants into two classes which was previously made on phenotypic grounds (chapter 4). Not only do the class II mutants show clear non-mendelian and non-chromosomal inheritance, but the determinant of the oligomycin resistance appears located on the mitochondrial DNA. The evidence for this conclusion has been presented in this chapter and is summarised below :

(i) Mitotic segregation is always observed in diploids obtained from crosses of the type $OL^{R}II\,\rho^{+}$ x $OL^{S}\rho^{+}$.

(ii) Tetrads from resistance and sensitive diploids give respectively 4 :0 and 0 :4 resistant :sensitive spores.

(iii) Recombination of the oligomycin resistant determinant(s) with other known mitochondrial markers such as the loci for chloramphenicol and erythromycin resistance has been observed (Table 6.19).

(iv) Diploids from crosses of the type $OL^{R}II\,\rho^{+}$ x $OL^{S}\rho^{-}$ fail to show mitotic segregation for oligomycin resistance.

(v) Diploids from crosses of the type $OL^{R}II\,\rho^{-}$ x $OL^{S}\rho^{+}$

fail to show mitotic segregation for oligomycin resistance.

The last observation was not found to hold in the case of strain D22A21. The reason for this is not known as in all other respects this strain behaves in quite an orthodox fashion. The discrepancy is not considered significant as firstly, the number of petites tested was small, secondly it is unknown whether the petites were cytoplasmic or nuclear and thirdly, it is known that the deletion of a particular gene(s) does not always accompany the formation of the ρ^- genotype (Linnane and Haslam, 1970; Slonimski, private communication). The class II mutants, therefore, resemble, at least en masse, the oligomycin resistant mutants isolated by Wakabayashi and Gunge, 1970; Stuart, 1970) - results reported almost simultaneously with the preliminary report of our own analyses (Avner and Griffiths, 1970).

Tables 6.14 and 6.15 show that within this group of mutants there are two groups of non-allelic mutants. The relationship of these two non-allelic groups to the mutants of Wakabayashi and Gunge and Stuart is unknown. Experiments conducted in collaboration with Professor Slonimski have, however, shown that the mutants in group 'R' are allelic to their oligomycin resistant determinants O^R1, O^R2, O^R4 and O^R7. The failure of either Stuart (Stuart, 1970) or Wakabayashi and Gunge (Wakabayashi and Gunge, 1970) to observe oligomycin resistance at the mitochondrial level in vitro may suggest that these workers have isolated yet another type of cytoplasmic oligomycin mutant, as mitochondria from all the class II mutants so far examined have shown resistance to oligomycin in vitro (J. R. Turner, private communication). In view of the difficulties intrinsic to the biochemical analysis of oligomycin resistance, the discrepancy may be more mere apparent than real.

Tzagaloff has isolated four proteins which appear to be associated with conferring oligomycin resistance on the ATPase in addition to the major proteins such as O.S.C.P. These proteins whose synthesis is chloramphenicol sensitive, are present when rutamycin sensitive ATPase preparations are subjected to gel electrophoresis but are absent from preparations which are rutamycin insensitive (Tzagaloff and Meagher, 1972; Tzagaloff, private communication). The finding of at least two non-allelic groups of oligomycin resistance determinants makes this report of especial interest.

The class I mutants are clearly differentiated in all respects from the class II

mutants. Despite the many peculiarities in the phenotypes observed, these do appear to be nuclear mutants. Accepting this premise, which admittedly requires consolidating by analysis of further tetrads, leaves, however, the problem of explaining the many results reminiscent of mitotic segregation or phenotypic or genic instability. Four main explanations may be advanced for the anomalous behaviour of the class I mutants :

(i) The observations are due to instability in OL^R gene expression in the diploid state due to the mixed nucleoplasm or cytoplasm present in such diploids. This hypothesis envisages in other words, some form of sporadic 'switching' on and off of the OL^R allele within the diploid cell, whilst within the haploid, expression is stable.

(ii) The OL^R gene expression is, as in model (i), unstable in the diploid cell but solely due to the presence of an OL^S allele in the same cell. This explanation appears unlikely as it has already been shown that anomalous results are obtained even when two OL^R alleles are present in the same diploid (Table 6.13).

(iii) That the expression of the OL^R locus is stable in the diploid and the effects seen are solely due to the random distribution of the products of competing OL^S and OL^R alleles within the diploid cell to a small mitochondrial population ($\sim$ 50 mitochondria per cell). Some evidence for the later may be forthcoming from electron microscopic examination of the heterogeneity of mitochondria within diploids from $OL^R\rho^+ \times OL^S\rho^+$ crosses. That is whether each individual diploid cell has only OL^R mitochondria or a mixture of OL^R and OL^S mitochondria.

(iv) Mitotic recombination is occurring in the diploid and causing the observed phenotypic patterns. As (Table 6.22) it appears that altering the plating density and therefore cell contact does not affect the resistance transmission frequency, such recombination, if occurring, must be predominantly due to completely intracellular recombination rather than mitotic recombination involving at any point intercellular exchange of genetic material. The results shown in Figure 6.2. would appear difficult to explain on this model.

An observation of great importance is the markedly quantitative nature of the resistance loss in the class I mutants. This applies to both the loss of resistance to cold exposure in the haploid class Ia mutants, resistance being lost apparently more quickly at higher levels of oligomycin, and the resistance transmission values observed amongst diploids from $OL^{R}I\rho^{+} \times OL^{S}\rho^{+}$ crosses. Both these results suggest that some dosage/resistance effect is occurring with possible titration of oligomycin resistant 'factors' by the drug itself. Clarification of the mode of inheritance in these mutants will be of great importance if their loss of resistance on exposure to low temperatures proves valuable for biochemical studies.

CHAPTER 7

WHOLE CELL STUDIES OF D6, D22 AND DERIVED OLIGOMYCIN RESISTANT STRAINS

Introduction

The demonstration of drug resistance in plating experiments may or may not be fully reproducible in liquid medium as shown of oxathiin resistance in Ustilago maydis (Georgopoulos and Sisler, 1970). Amongst other differences between experiments performed in solid and liquid medium is the time scale over which observations are made during the experiments. Plating assays are generally scored after 2 - 3 days growth, whilst assaying for drug resistance by the addition of a drug to logarithmic phase cultures permits observations on at least an hourly time scale. Thus, strains in which drug resistance is not due to a constitutive enzyme activity, but an inducible one, may appear completely resistant on plating experiments, whilst showing a time lag before growth is resumed when resistance assays are performed in liquid medium. This is especially true in the case of mutants where the inducible effect is one conferring resistance to a drug which affects a major metabolic process. For instance, if CCP resistance in resistant mutants is due to an inducible enzyme activity requiring some type of de novo synthesis, then on addition of the drug to non-induced cultures growing on a non-fermentable medium, its primary effect will be the same as that on a sensitive cell - to shut down all oxidative phosphorylation. Similar effects will also be caused by antimycin A and oligomycin, except in the latter case only the production of ATP and not of all high energy intermediates will be interfered with (Kaniuga, et al., 1969; Slater and Ter Welles, 1969). This primary effect means that all synthetic activities requiring energy and necessary for the induction of resistance must be funded either from pre-existing ATP and energetic intermediates pools within the cell, or from any fermentable substrate pools formed from non-fermentable substrates during the course of normal cellular metabolism, or from any 'leaks' in the drug inhibition which allow 'high energy' formation by mitochondrial respiratory activity. Under these conditions the cellular energy supply may well determine the rate of adaptation to the drug and therefore the speed at

which cellular growth is resumed. This may obviously involve a comparatively long time, so that although the strain appears to grow in the presence of the drug when growth is measured as in plating experiments over 72 hours, it will not, over say a 12-hour experiment in liquid medium. The effects mentioned using chloro CCP, antimycin A and oligomycin as examples may be especially severe in the case of chloro CCP as it may, like other uncouplers (Harary and Slater, 1965), cause the hydrolysis of preformed ATP pools within the cell (Slater and Ter Welles, 1969). Similar examples may be constructed in the case of antibiotics which inhibit protein synthesis and may be expected to directly inhibit the synthesis of any inducible protein responsible for resistance.

A further difference, apart from the time scale, between plate and liquid culture experiments is the metabolic state of the cells used in the experiments. In liquid drug resistance assays the cells have been grown in the presence of non fermentable substrates for several generations whilst in the former case, cells are generally taken from YEPG slopes or plates of an age which has not been closely determined. This might mean, on the basis of the model discussed above, that there is a much greater pool of endogeneous fermentable substrate in cells used for plating experiments than for liquid ones, and this will obviously affect any adaptation process. Furthermore, the difference in the conditions under which the cells were grown may well affect the lipid composition of the cells (for references see chapter 5). In view of the lipophilicity of many of these inhibitors including oligomycin (for references see chapter 4) and observations such as the increased amounts of glycoproteins, lipoproteins and glycolipids present in actinomycin D resistant cell lines compared with actinomycin D sensitive cell lines (Bosmann, 1971), the postulated lipoprotein nature of the DCCD binding site (Cattell et al., 1971), and the effect of uncouplers such as chloro CCP in interacting with cardiolipin and stabilising its turnover (Ono and White, 1971), if the growth conditions did affect lipid composition, such an effect could also explain non-correlated findings in plating and liquid resistance assays.

A further difference between the two types of assay concerns the relative immobility of the drug in plating experiments. It is, for instance, far easier to imagine an efficient detoxifying mechanism working in solid medium where diffusion of the drug into the cell may quickly become limiting than it is in liquid medium

where such diffusion would not be expected to become limiting so quickly. This type of explanation assumes that the detoxification mechanism, if it exists, has not a high enough turnover rate to cope with the inhibitor efficiently, when the inhibitor can freely diffuse into the cell at concentrations equal to those used originally in the plating medium. The two types of assay may also vary in the ratio of drug concentration per cell even though the absolute drug molarities used are the same in both cases. Discrepancies due to this cause, however, should be easily resolvable because in some ranges of drug concentrations, discrimination of the resistant from the sensitive strains should be possible, even if the absolute molarities used in the two types of experiments are thereby rendered very different.

The experiments described in this chapter are concerned with the analysis at the level of the whole cell of the physiological characteristics of the different types of mutation conferring oligomycin resistance. The first group of results presented relate to experiments designed to demonstrate the oligomycin resistance or otherwise of the oligomycin resistant mutants in liquid medium. Such a demonstration is of crucial importance for two reasons. Firstly, if the resistance is 'inducible' then biochemical analysis is unfeasible as it is not practicable to grow large scale yeast cultures in the presence of oligomycin. Secondly, if the resistance is not expressed constitutively, the chances of it being due to changes in the organisation of the mitochondrial membranes and, more specifically, the ATP synthetase complex itself would appear remote, thus considerably reducing its intrinsic biochemical interest. As attempts to demonstrate inhibition of intact cell respiration by oligomycin have met with only slight success, it was not possible to examine the mutants in an oxygen electrode for their resistance to oligomycin. Attempts have, however, been made to correlate the oligomycin resistance of the various strains to their resistance to DCCD inhibition of intact cell respiration.

Resistance in liquid medium or in an oxygen electrode to oligomycin even if demonstrated, however, provides no information as to whether the mutation is due to a change in cell permeability, mitochondrial permeability, or due to a more fundamental change at the actual locus of action of the drug. In the absence of the possibility of measuring isotopically the uptake of oligomycin into the various strains, attempts have been made to utilise the ability of oligomycin to induce petites in order to identify which, if any, of the resistant strains was still

permeable to oligomycin. If, for instance, any or all of the OL^R strains still showed the petite induction effect caused by oligomycin, then it would be reasonable to assume that the oligomycin was still entering the cell, and that therefore the resistance was not due to an alteration in cell permeability. The alternative finding that petite induction did not occur, however, permits of no conclusion, as resistance to oligomycin due to changes at either the cell or mitochondrial level might result with equal efficiency.

The fourth group of experiments related to whether any of the oligomycin resistant mutants shows an alteration in either growth rate or growth yield. The observation of such characteristics in otherwise isogenic strains might provide good evidence of changes in the strain at the mitochondrial level. An electron microscopic investigation of the mutants has also been undertaken. Although in view of the isolation procedure and the subsequent finding that neither the class I or class II mutants differ in their growth rates, gross changes in mitochondrial structure such as have been observed in petite strains were not envisaged (Avers et al., 1965; Smith et al., 1969; Yotsuyanagi, 1962; Federman and Avers, 1967) investigation of the mitochondrial structure of these mutants appeared warranted.

Results

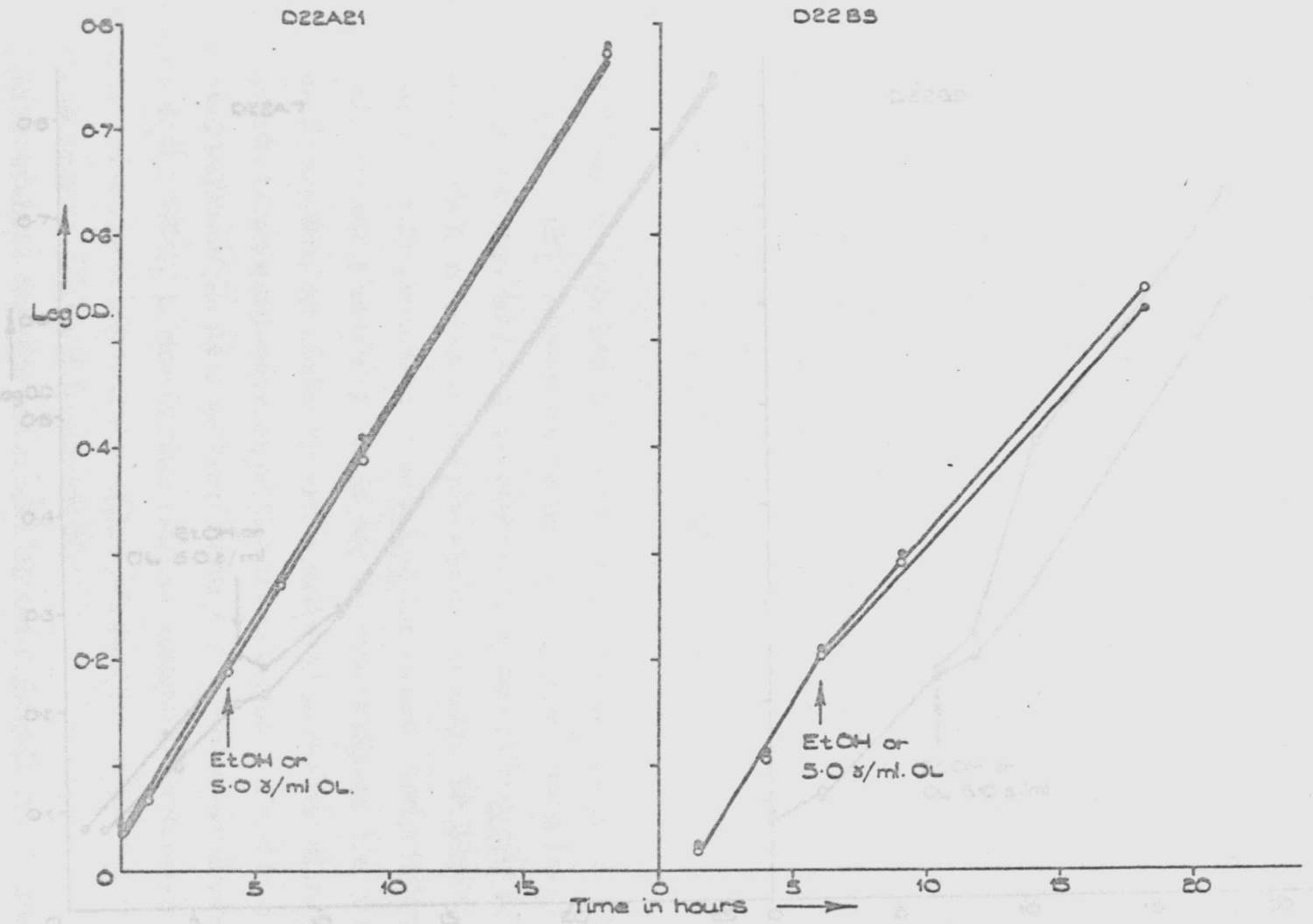
Response of the oligomycin resistant mutants to oligomycin in liquid medium

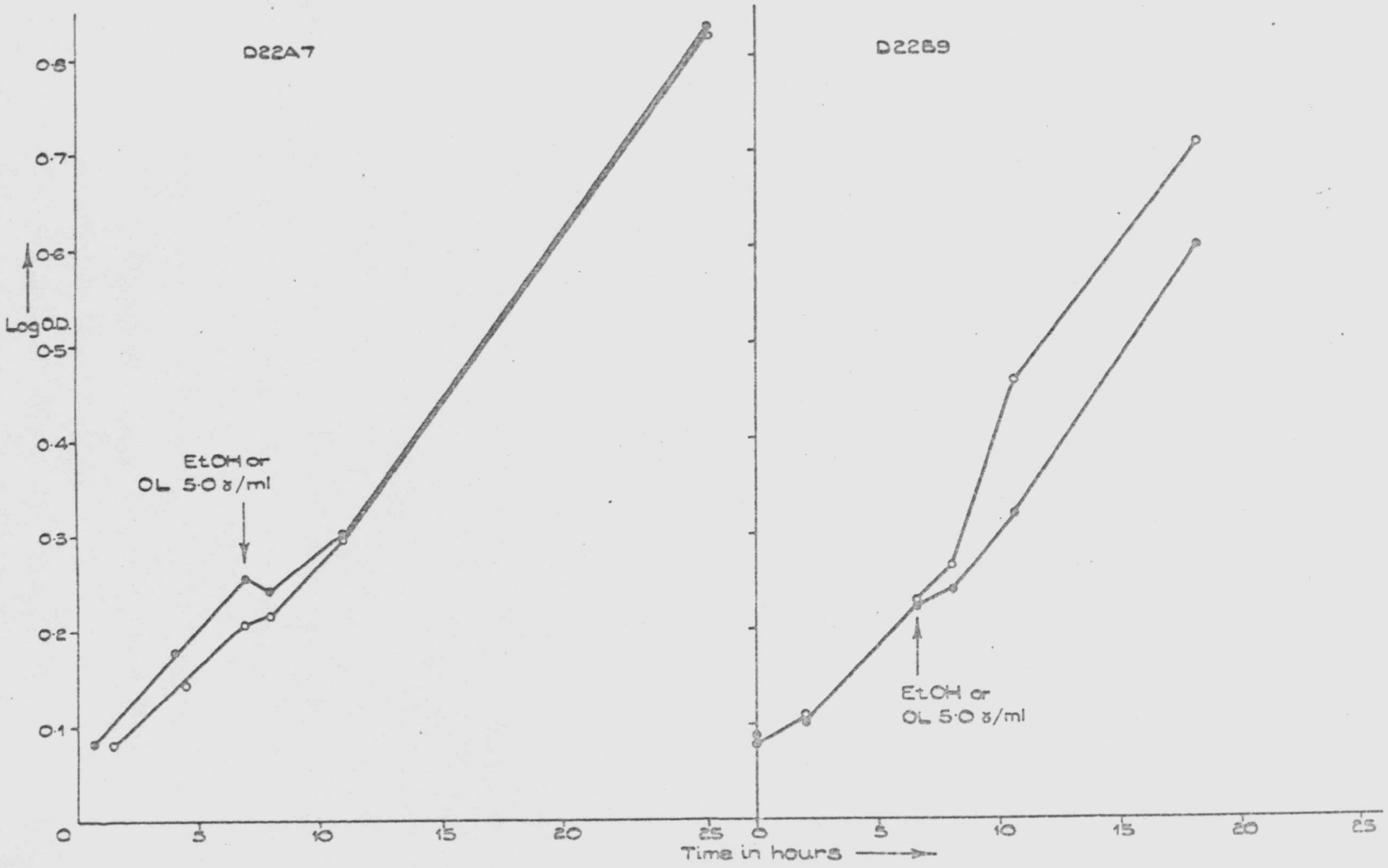
The response of the wild type strain D22 to oligomycin when grown on fermentable and non-fermentable medium has been discussed in chapter 4. As shown in those results, addition of oligomycin to mid-log phase cells of D22 growing on non-fermentable medium results in an almost immediate inhibition of growth. In contrast, none of the D22 OL^R strains tested, regardless of whether they were of the class I or class II type, was inhibited by oligomycin under identical conditions. Figures 7.1. and 7.2. present examples of the results obtained. The strains examined were D22 A4, D22 A7, D22 A21, D22 B3 and D22 B9. In a few cases when the cells were growing with glycerol rather than ethanol as the carbon source, oligomycin seemed to reduce in whole or in part the stimulation of the growth rate observed on addition of small amounts of ethanol to glycerol grown cultures. A systematic examination of the conditions governing this response has not, however, been carried

LEGEND TO FIGURES 7.1. AND 7.2.

Oligomycin Resistance of OL^R Strains in Liquid Medium

Standard inocula (0.5% - 0.65%) of the various strains were added to 500 ml sidearm flasks containing 25 ml of medium. The inocula used were all taken from stationary phase YEPG grown cultures. The flasks were incubated on an orbital shaker at 30°C and 5.0 γ/ml oligomycin were added as an ethanolic solution at the points indicated on the graph when the cultures were in mid-log phase. Equivalent amounts of ethanol were added to the control flasks at the same time. The growth of the cultures was followed using an EEL colorimeter fitted with a 607 filter (red).


D22A21
D22B3
0·8
0·7
0·6
0·4
0·2
0
Log O.D.
5
10
15
0
5
10
15
20
Time in hours
EtOH or
5·0 γ/ml OL.
EtOH or
5·0 γ/ml. OL


D22A7
D2289
Log O.D.
0·8
0·7
0·6
0·5
0·4
0·3
0·2
0·1
0
EtOH or
OL 5·0 γ/ml
EtOH or
OL 5·0 γ/ml
0
5
10
15
20
25
Time in hours

out and it is not known whether such a response is characteristic of the class I mutants alone or not.

The results clearly show that there is no discernible lag in the phenotypic expression of oligomycin resistance on addition of the drug to mid-log phase cells, any slight cessation of growth being no greater than that produced in the control cultures by the addition of ethanol alone.

Effect of DCCD on the respiration of intact yeast cells

Attempts to demonstrate oligomycin inhibition of intact cell respiration failed to give any significant inhibition in our hands, even when high concentrations of the drug were used. Kovác et al., whilst finding a small inhibition of yeast cell respiration, also had to use high oligomycin concentrations and found that this inhibition could not always be demonstrated (Kovác et al., 1970). Even in tissue slice experiments where permeation of oligomycin into the cell should pose a less severe problem, oligomycin inhibited respiration of frog muscle, rat diaphragm, brain and kidney cells by no more than 30% (Tobin and Slater, 1965). Similar results have been found by other workers (Van Rossum, 1964, 1967; Whittam et al., 1964) whilst Dallner and Ernster, and Minakami et al. found much greater inhibitions of respiration in ascites tumour cells (Dallner and Ernster, 1962; Minakami et al., 1963). Whether the low inhibition rates of respiration by oligomycin reported truly represent the loosely coupled nature of most cells in vivo or an in vitro experimental artifact, is not clear (see also Currie and Gregg, 1965) but if the former is true then some cells are more coupled in vivo than others. Such a result for tumour cells, in view of their high growth rate, is perhaps logical, i.e. they have to be normally coupled, but equally the recent finding of the absence of inducible ATPase activity in many hepatomas might lead one to suggest that these tissues stand a much improved chance of not being 'uncoupled' by experimental artifacts (Pedersen et al., 1971). In view of these results, attempts to demonstrate differential resistance of respiration to oligomycin inhibition in D22 and derived OL^R strains seemed unlikely to be successful.

As the evidence suggests that both DCCD and oligomycin inhibit mitochondrial oxidative phosphorylation at closely related, if not identical, sites (see chapter 1 for references) and Kovác et al. had shown that DCCD inhibits yeast cell respiration, the effects of the latter agent on the respiration of D22 and the derived OL^R mutants

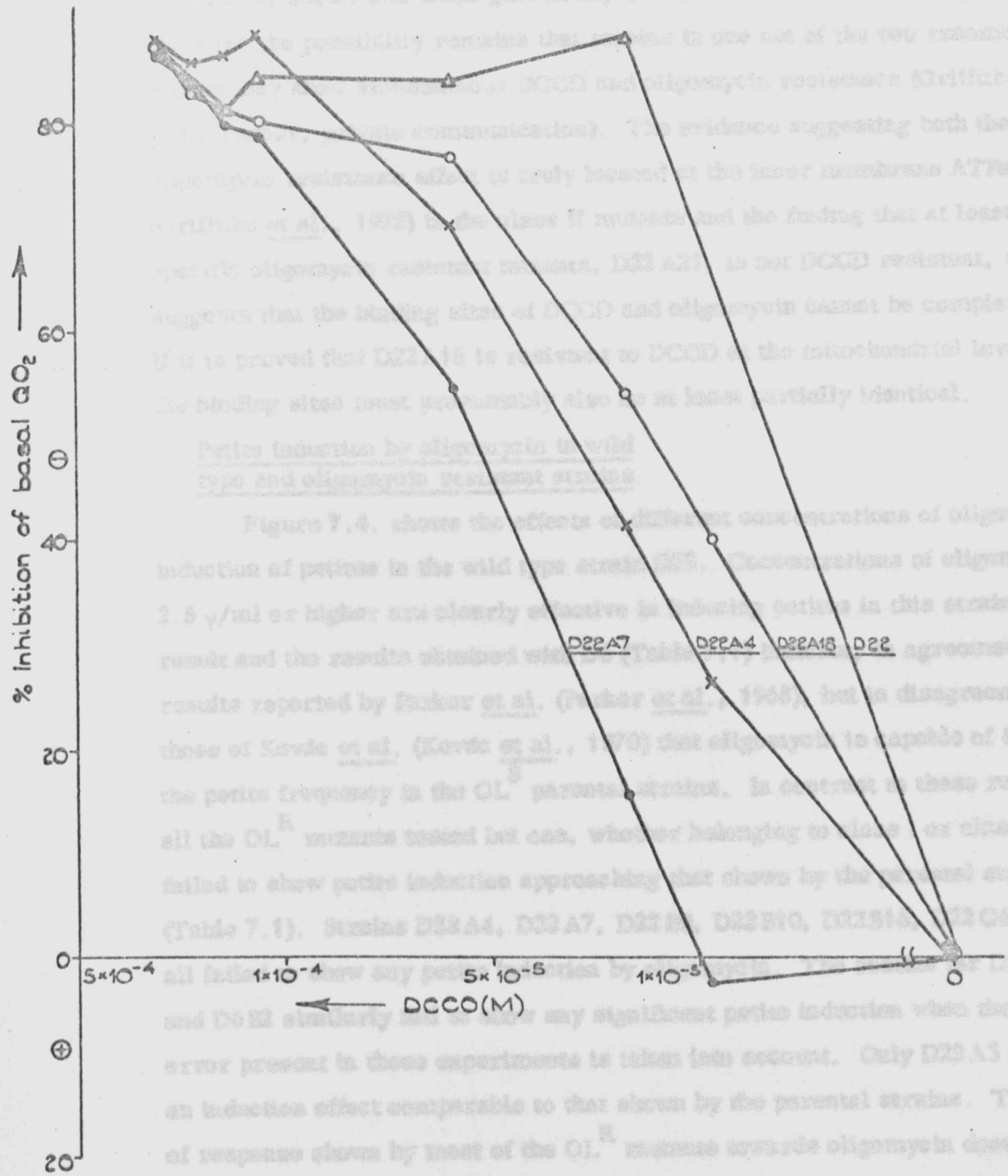
has been examined (Kovác et al., 1968b). Although the long term plating experiments (chapter 4) had been unsuccessful, short term respiration assays will not have many of the disadvantages in terms of DCCD instability, etc. that characterised the former experiments. Kovác et al. had, moreover, shown that the respiration inhibition seen in short term experiments (i.e. 0 - 120 seconds after the addition of DCCD) was relievable by FCCP so that the in vivo action of DCCD within this time scale anyway appears superficially the same as that characteristic of the in vitro mitochondrial inhibition (Kovác et al., 1968b). In preliminary experiments, in agreement with Kovác et al. (Kovác et al., 1968b), DCCD was found to inhibit the respiration of the wild type strain, D22 (Figure 7.3). A consistently higher inhibition of respiration than Kovác et al. have reported was, however, found not only at given concentrations of DCCD, but also at saturating concentrations of the compound as well. As the experimental conditions used were the same, it may be that this difference is due to their use of partially glucose repressed cells. As no data on the growth conditions used by Kovác et al. (Kovác et al., 1968b) are available, verification of this is not possible. We have found, however, inhibition values close to those described by these workers in experiments with partially glucose repressed cells and the inhibition values in this case were not increased by the use of very high DCCD concentrations (3.0×10^{-4}M).

Figure 7.3. also shows the results obtained with three OL^R strains of yeast isogenic with D22 except for the OL^R gene. All were grown under identical regimes and all assays carried out at identical cell densities. Both the class I mutants D22A7 and D22A4 are clearly more resistant to DCCD than the wild type strain D22. This result is in agreement with the results in vitro where the ATPase activity of the mitochondria isolated from these strains was more resistant to DCCD inhibition than was that of mitochondria extracted from D22 (J.R.Turner, private communication). In these experiments, however, mitochondria from D22A4 appeared more resistant than those of D22A7 whilst the opposite apparently pertains in the intact cell. D22A18, the only class II mutant tested, also appears slightly more resistant than D22 but in this case the scatter of inhibition values found was such that it is not possible to be unequivocal. In connection with these experiments it should be pointed out that each point represents the mean of two or three determinations, each inhibition value being estimated 90 seconds after the addition

LEGEND TO FIGURE 7.3.

DCCD Inhibition of Intact Yeast Cell Respiration

The strains used, D22, D22A4, D22A7 and D22A18 were all grown on YEP 0.5% EtOH medium at 30°C until they reached an OD of 5.0 - 6.5 when they were harvested, washed once and resuspended in a small volume of 0.1 M phosphate buffer pH 7.0 + 2% glucose. The cell suspensions were kept in ice. Respiratory assays were carried out by adding 50 µl of the yeast suspension to 2.8 ml of citrate phosphate buffer pH 4.6 containing 2% glucose and 10 mM KCl. DCCD (100 µl aliquots) was added as an ethanolic solution and the respiratory rate (inhibited) estimated 90 seconds after the addition of the inhibitor. The percentage inhibition of the basal respiration rate was calculated.


% Inhibition of basal QO_2
80
60
40
20
0
20
5×10^{-4}
1×10^{-4}
5×10^{-5}
1×10^{-5}
0
DCCD(M)
D22A7
D22A4
D22A18
D22

of DCCD in order to avoid including in the estimate any DCCD inhibition which is not relievable by chloro CCP (Kovác et al., 1968b).

From this data it appears that at least two of the class I mutants are also DCCD resistant and possibly at least one of the class II. The only class II mutant, D22A21, tested at the mitochondrial level admittedly has shown little or no DCCD resistance, but as this maps genetically in a different recombination group from D22A18, the possibility remains that strains in one out of the two recombination groups may show simultaneous DCCD and oligomycin resistance (Griffiths et al., 1972; J. R. Turner, private communication). The evidence suggesting both that the oligomycin resistance effect is truly located at the inner membrane ATPase (Griffiths et al., 1972) in the class II mutants and the finding that at least one of the specific oligomycin resistant mutants, D22A21, is not DCCD resistant, strongly suggests that the binding sites of DCCD and oligomycin cannot be completely identical. If it is proved that D22A18 is resistant to DCCD at the mitochondrial level, then the binding sites must presumably also be at least partially identical.

Petite induction by oligomycin in wild type and oligomycin resistant strains

Figure 7.4. shows the effects of different concentrations of oligomycin on the induction of petites in the wild type strain D22. Concentrations of oligomycin of 2.5 γ/ml or higher are clearly effective in inducing petites in this strain. This result and the results obtained with D6 (Table 7.1) indicate, in agreement with the results reported by Parker et al. (Parker et al., 1968), but in disagreement with those of Kovác et al. (Kovác et al., 1970) that oligomycin is capable of increasing the petite frequency in the OL^S parental strains. In contrast to these results, all the OL^R mutants tested but one, whether belonging to class I or class II, failed to show petite induction approaching that shown by the parental strains (Table 7.1). Strains D22A4, D22A7, D22B9, D22B10, D22B16, D22C4 and D6A1 all failed to show any petite induction by oligomycin. The results for D22A15 and D6B2 similarly fail to show any significant petite induction when the sampling error present in these experiments is taken into account. Only D22A5 showed an induction effect comparable to that shown by the parental strains. The lack of response shown by most of the OL^R mutants towards oligomycin does not seem to be particular to the synthetic medium used in these experiments as D22A4, when grown in YEPG medium with oligomycin (5.0 γ/ml), still shows no petite induction. The finding of petite induction by oligomycin in D22 and D6 in both

LEGEND TO FIGURE 7.4.

Effect of Oligomycin on the Petite Frequency of D22

A 2% inoculum of a D22 culture which had been grown overnight in YEPG medium was added to flasks containing fresh YEPG medium together with various concentrations of oligomycin. The cultures were grown at 30°C for 42 hours when they were analysed for petite frequency by plating onto PDM medium. These plates were scored after 5 days incubation at 30°C. The concentrations of oligomycin tested were 0, 0.5, 2.5, 5.0, 7.5 and 10.0 γ/ml. The final EtOH concentration in the medium in all cases was 0.5%.

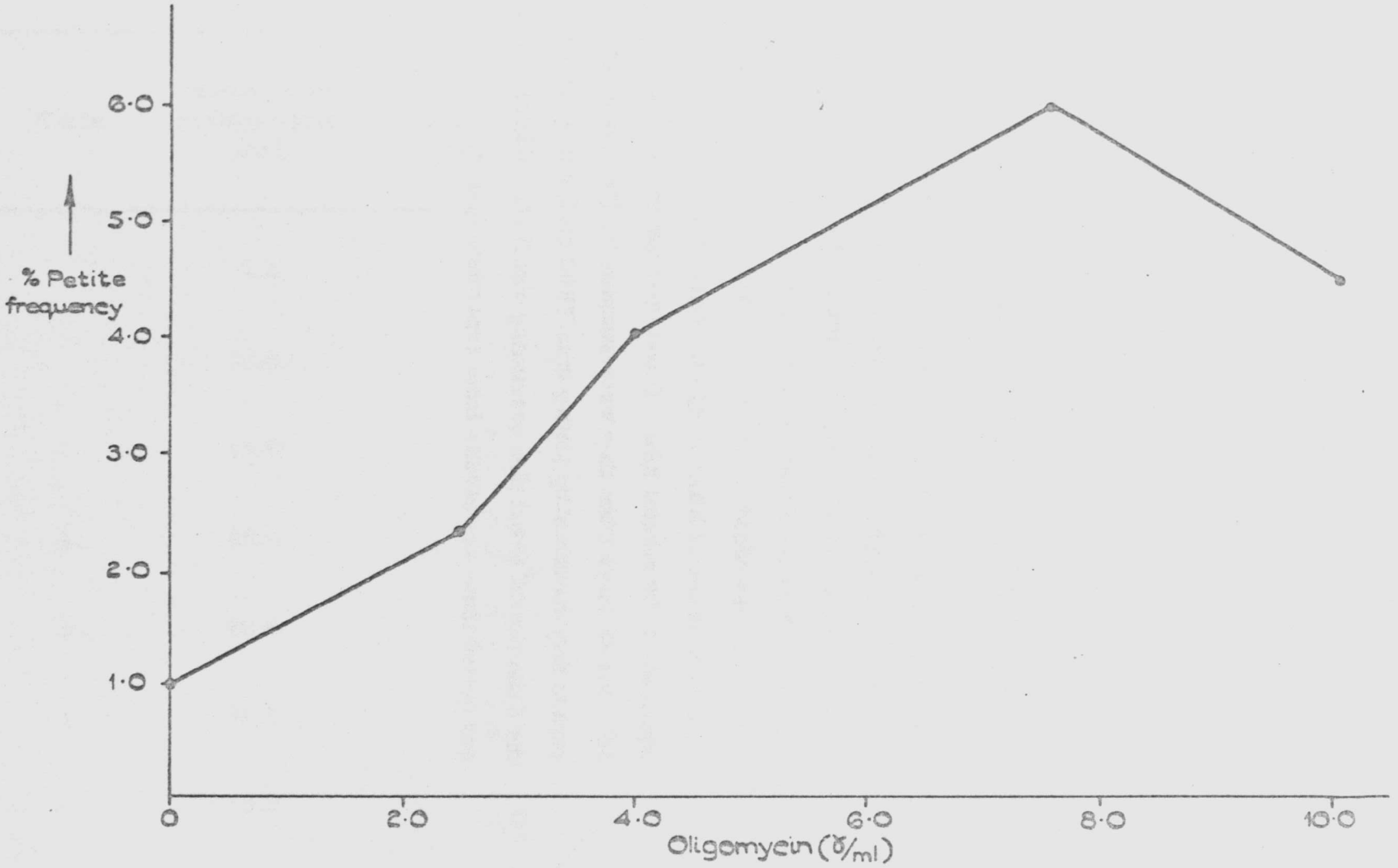

6·0
5·0
% Petite frequency
4·0
3·0
2·0
1·0
0
2·0
4·0
6·0
8·0
10·0
Oligomycin (γ/ml)

TABLE 7.1.

Petite Induction by Oligomycin in OL^S and OL^R Strains

The strains to be used were grown in liquid YEPG medium until they were in mid-glycolytic log phase ($^{\Delta}$OD 2.5) when a 1% inoculum of each was put into shake tubes containing 10 ml of Wickerhams glucose medium supplemented with adenine, arginine and methionine. Oligomycin was added at the concentrations noted in this table and the concentration of EtOH so added, which was never above 1%, was equalised in the control tube. Tubes were grown with shaking at 30°C for 48 hours when they were sampled for the percentage petites they contained by plating onto YEPG medium, incubating for 3 days at 30°C and then overlaying with TTC. Where 2 values are quoted these are results from separate cultures.

TABLE 7.1.

Strain	Class	Concentration of Oligomycin γ/ml	Percentage Petite Frequency - Oligomycin	Percentage Petite Frequency + Oligomycin	Percentage Mean Change in Petite Frequency ± Oligomycin	Total No. of Cells Analysed - Oligomycin	Total No. of Cells Analysed + Oligomycin
D6		5.0	15.6 21.0	42.0 55.0	+ 170	128 229	431 856
D22		10.0	1.4 1.9	8.2	+ 400	796 425	442
D22A4	I	10.0	4.1 3.6	2.8 5.0	0	147 333	143 177
D22A5	I	10.0	0.5 0.5	1.9 1.7	+ 260	208 260	417 394
D22A7 (a)	I	10.0	2.2 1.8	1.3 2.2	-15	315 381	481 504
D22A7 (b)	I	10.0	0.5 0.9	0.4 0.8	- 10	584 557	288 253
D22A15	II	10.0	0 2.0	2.8 0.5	+ 65	157 350	279 252

continued .....

TABLE 7.1. (continued)

Strain	Class	Concentration of Oligomycin γ/ml	Percentage Petite Frequency - Oligomycin	Percentage Petite Frequency + Oligomycin	Percentage Mean Change in Petite Frequency ± Oligomycin	Total No. of Cells Analysed	
						- Oligomycin	+ Oligomycin
D22B10	I	5.0	1.6 2.2	0.6 1.5	- 45	254 418	324 326
D22B9	I	10.0	0.4 1.0	0.8 0.7	+ 9	1351 800	1274 1054
D22B16	I	10.0	1.9	2.7 1.1	0	316	511 528
D22C4	II	10.0	1.3 2.1	1.7 1.3	+ 17	383 467	284 230
D6A1	II	2.5	24.2 25.2	21.5	- 13	256 384	530
D6B2	II	5.0	8.8 5.5	11.1 13.3	+ 70	513 144	198 293
D6B2	II	10.0	5.0	4.3	- 14	228	100

YEPG and synthetic medium similarly supports this conclusion.

On the basis of these results it is impossible to conclude whether oligomycin is entering the cells of the OL^R mutants or not, as both cell permeability and changes in the mitochondria could cause, with equal efficacy, the observed results. Only in the case of D22A5 is one able to conclude that the cell is still permeable to oligomycin. There existed the possibility that the lack of response of the petite frequency of the OL^R mutants to oligomycin was a function of the growth period at which sampling was carried out. In the experiments with the parental strains D22 and D6 which show petite induction, for instance, the comparison made between the control and oligomycin treated cultures (Table 7.1.) is actually a comparison between a stationary oxidative phase culture without oligomycin and a stationary glycolytic phase culture containing oligomycin, as the presence of oligomycin will inhibit the oxidative phase of growth of these strains. On the other hand, both the oligomycin treated and control cultures of the OL^R strains were sampled at the end of the oxidative growth phase as oligomycin is without an inhibitory effect on these strains. In order to eliminate this difference in procedure, as the cause of the difference in results obtained with the OL^S and OL^R strains, the latter were grown with and without oligomycin and sampled for the percentage of petites at the end of both the glycolytic and oxidative growth phases. The results are shown in Table 7.2. In none of the cultures tested was any oligomycin induced change in petite frequency approximating to that found with both D22 and D6 noted. This conclusion held regardless of whether the cells were sampled at the end of the glycolytic phase before oxidative growth commenced, or at the end of this oxidative phase of growth. This is especially clearly shown by the result with D6A1 where there is little sampling error as the spontaneous petite frequency is high. It seems fair to conclude, therefore, that oligomycin is genuinely unable to induce petite mutation in virtually all the oligomycin resistant mutants.

Characterisation of the growth rate of D22, D6 and the derived OL^R mutants

Preliminary experiments indicated that the growth rate of the strains grown on YEP medium was higher when 1% ethanol was the carbon source than when 2% ethanol served this function. Figure 7.5. shows that, in fact, the growth rate increases as the ethanol content of the medium decreased to 0.25% and this experiment, in conjunction with others (not shown), established that there is an

TABLE 7.2.

Effects of Growth Phase on Petite Induction by Oligomycin

1% inocula of YEPG grown mid-log phase ($^{\Delta}$OD 2.5) cells were added to 100 ml of YEPG $\pm$ oligomycin (concentration as shown in Table 7.2) in 500 ml sidearm flasks and the flasks incubated at 30°C. Growth was followed using an EEL colorimeter (607 filter). At the end of the glycolytic and oxidative phases of growth, samples were taken from the oligomycin treated and control flasks and plated onto YEPG medium and grown at 30°C, following which the petite frequency was estimated using a TTC overlay.

Numbers in parentheses = total number of cells analysed.

TABLE 7.2.

Strain	Class	Oligomycin Concentration γ/ml	Percentage Petite Frequency - OL Glycolytic Phase	Percentage Petite Frequency + OL Glycolytic Phase	Percentage Petite Frequency - OL Oxidative Phase	Percentage Petite Frequency + OL Oxidative Phase
D6 A1	II	2.5	21.7 (1078)	7.2 (1360) 8.2 (736)	7.6 (890) 7.8 (790)	8.7 (2727) 8.5 (1626)
D22 A7	I	10.0	0.3 (1317)	0.5 (1314) 0.7 (1467)	1.5 (462)	1.0 (488)
D22 C4	II	10.0	0.8 (1357)	0.6 (990)	0.6 (506)	0.5 (200)

LEGEND TO FIGURE 7.5.

Effects of Ethanol Concentration on the Growth Rate of S.cerevisiae

Flasks containing 100 ml of standard YEP medium had various amounts of ethanol added to them after autoclaving. After addition of a standard inoculum of a stationary phase culture of D22A4 the flasks were grown on a Gallenkamp orbital shaker at 26°C (speed 250 rev/min) and growth of the cultures followed using an EEL colorimeter fitted with a 607 filter.

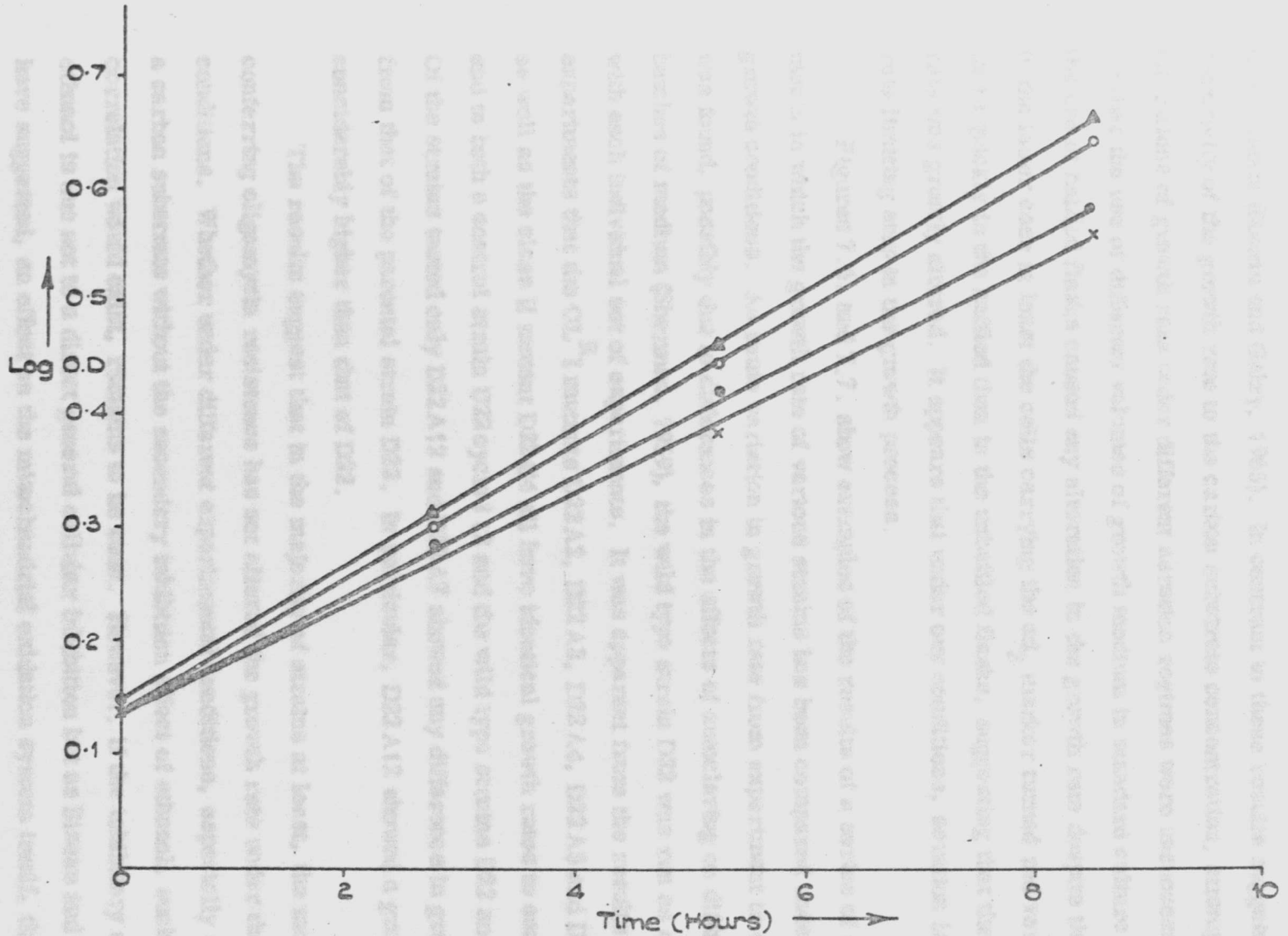
Key

× YEP 1% EtOH

• YEP 0.75% EtOH

○ YEP 0.50% EtOH

▲ YEP 0.25% EtOH


0·7
0·6
0·5
0·4
0·3
0·2
0·1
Log O.D
0
2
4
6
8
10
Time (Hours)

optimum ethanol concentrations of around 0.25%. The reason for the apparent growth retarding effects of higher ethanol concentrations which, however, are still comparatively low, may be connected with the finding by Bizeau and Galzy that high concentrations of ethanol (190 mM) inhibit yeast respiratory activity in short term experiments (Bizeau and Galzy, 1965). In contrast to these results suggesting the sensitivity of the growth rate to the carbon substrate concentration, attempts to show alterations of growth rate under different aeration regimes were unsuccessful. Neither the use of different volumes of growth medium in standard culture flasks nor the use of baffled flasks caused any alteration in the growth rate despite the fact that in the latter case at least the cells carrying the ad_2 marker turned red very much more quickly in the baffled than in the unbaffled flasks, suggesting that the aeration rate was greatly altered. It appears that under our conditions, aeration is not the rate limiting step in the growth process.

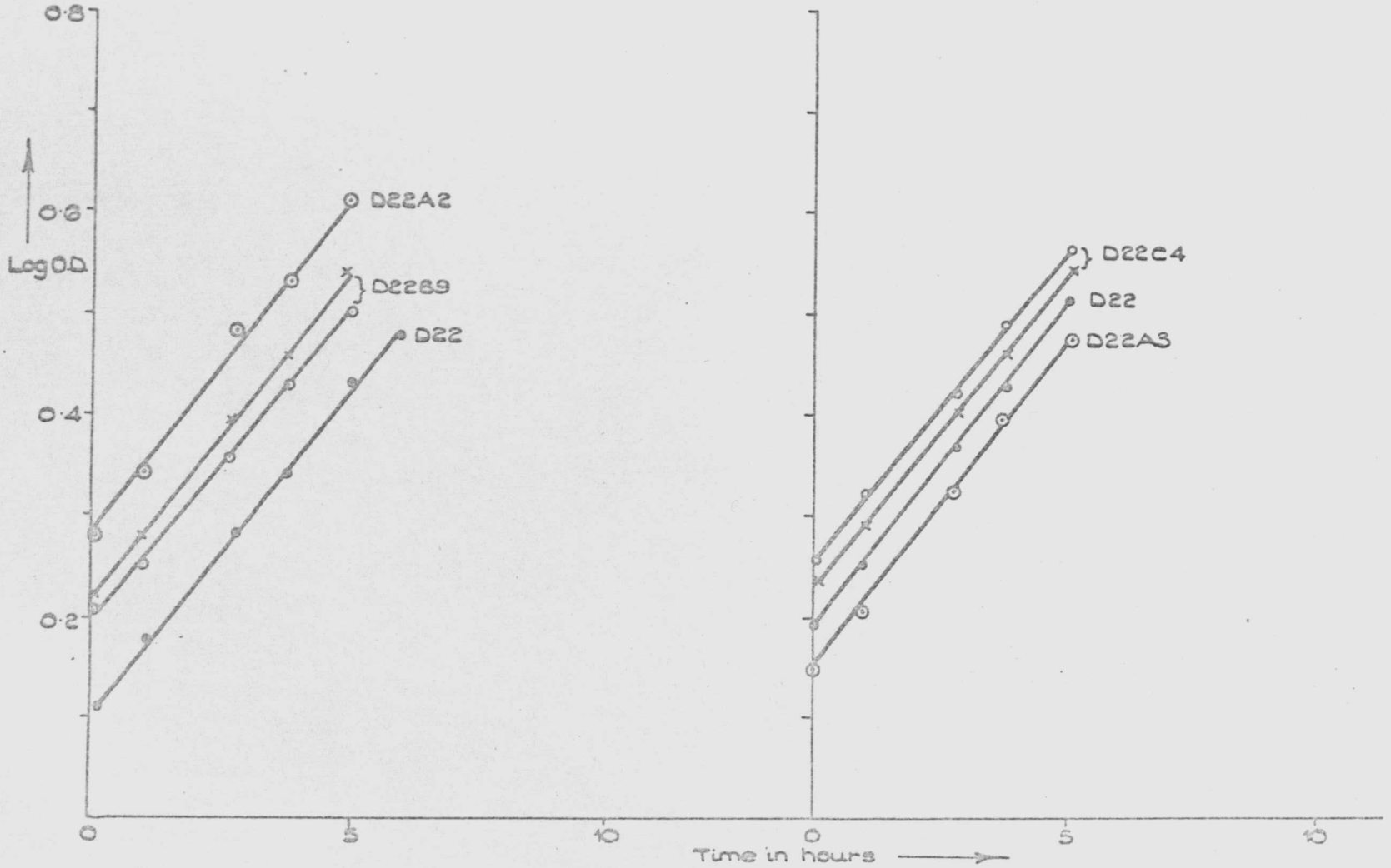
Figures 7.6. and 7.7. show examples of the results of a series of experiments in which the growth rate of various strains has been compared under identical growth conditions. As some variation in growth rate from experiment to experiment was found, possibly due to differences in the effects of autoclaving on different batches of medium (Sherman, 1959), the wild type strain D22 was run as a control with each individual set of experiments. It was apparent from the results of these experiments that the $OL^{R}I$ mutants D22A2, D22A3, D22A4, D22A5 and D22B9 as well as the class II mutant D22C4 all have identical growth rates to each other and to both a control strain D22cyclo 1 :2 and the wild type strains D22 and D6. Of the strains tested only D22A12 and D22A7 showed any difference in growth rate from that of the parental strain D22. In particular, D22A12 showed a growth rate considerably higher than that of D22.

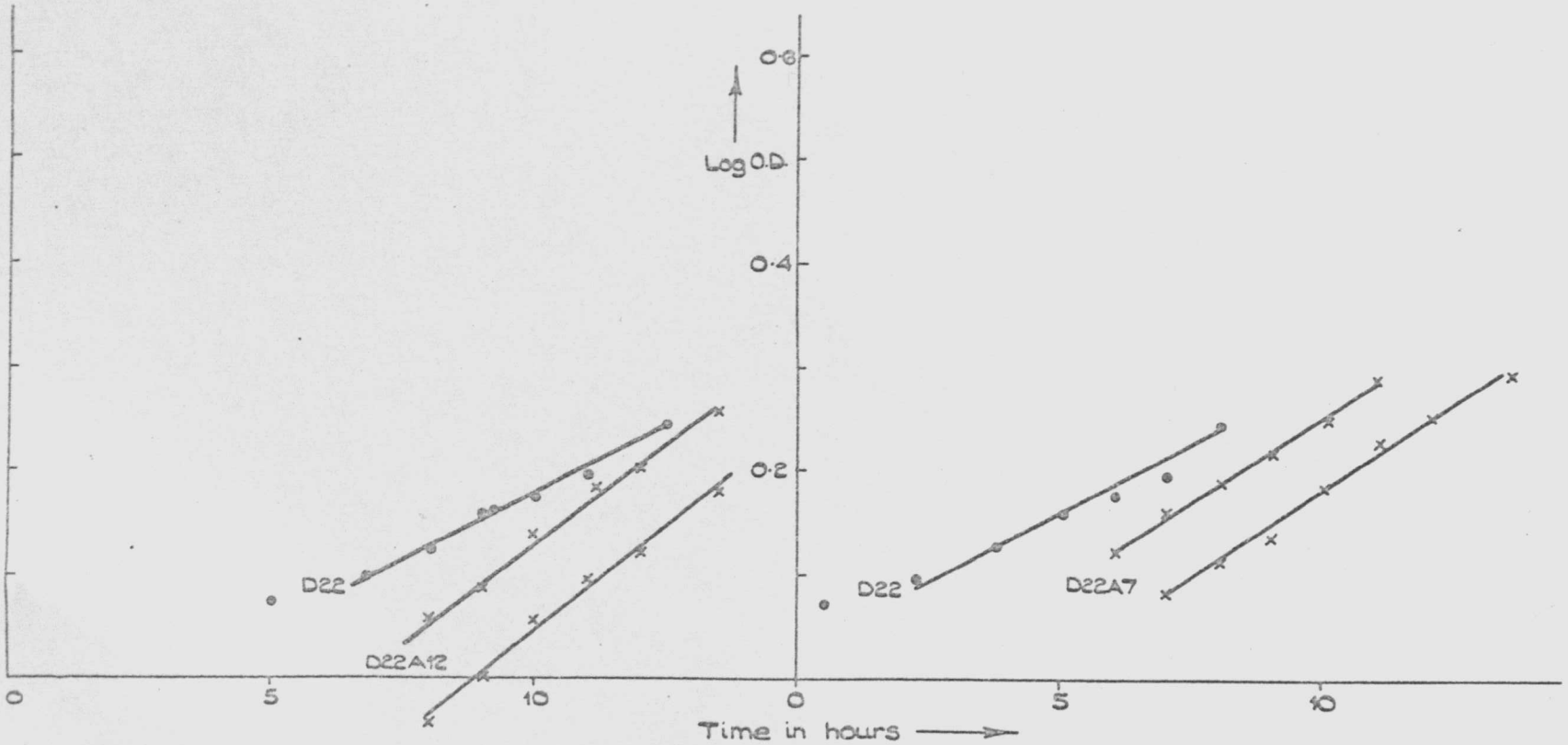
The results suggest that in the majority of strains at least, the mutation conferring oligomycin resistance has not altered the growth rate under these test conditions. Whether under different experimental conditions, especially using a carbon substrate without the secondary inhibition effect of ethanol, such a correlation would exist, remains to be seen. However, if the inhibitory effect of ethanol is due not to a direct general cellular inhibition but as Bizeau and Galzy have suggested, an effect on the mitochondrial oxidation system itself, then if all

LEGEND TO FIGURES 7.6. AND 7.7.

Analysis of the Growth Rate of D22 and D22 OL^R Mutants

The various strains were grown on 100 ml of YEP medium containing 0.5% ethanol in 500 ml sidearm conical flasks. Cultures were grown at $30^{\circ}C$ and the growth of the cultures during the logarithmic phase measured using an EEL colorimeter fitted with a 607 filter.


0·8
0·6
0·4
0·2
Log OD
D22A2
D22B9
D22
0
5
10
D22C4
D22
D22A3
0
5
10
Time in hours


0·6
Log O.D.
0·4
0·2
D22
D22A12
D22
D22A7
0
5
10
0
5
10
Time in hours

the strains have an unaltered response to this inhibitory action, the results may still represent the relative growth rate of the strains under limiting conditions of carbon substrate utilisation (Bizeau and Galzy, 1965).

Growth yield analysis of strains D6, D22 and derived OL^R mutants

Figure 7.8. shows the result obtained when the growth yield of the wild type strains D6 and D22 were measured using a range of ethanol concentrations. Although the intercept is different for the two strains, the slopes are identical so that the $Y_{substrate}$ calculated from these slopes is also identical. The presence in all these experiments of a small amount of cell growth in the absence of any carbon source is presumably due to energy derived from the yeast extract and peptone present in the basal medium. The $Y_{substrate}$ calculated for both the wild type strains D22 and D6 (Table 7.3) has a mean value of 42.0. This is considerably lower than that reported by Kormancikova et al. (Kormancikova et al., 1969) who reported a value of 70.0 for their strain when grown on ethanol. Our result is, however, in close agreement with the results of Hernandez and Johnson who found a $Y_{substrate}$ of 41.5 for C. utilis grown in an enriched medium with ethanol as the energy source (Hernandez and Johnson, 1967). A similar $Y_{substrate}$ value was also found for D22 and D6 when a synthetic medium containing a salt mix supplemented only with arginine, methionine and adenine was used (Wickerham's minimal medium + supplements), though in this case the value was fractionally higher (Table 7.3). In view of the finding by Kormancikova et al. (Kormancikova et al., 1969) that aeration affected the growth yield, attempts were made to try to increase the growth yield by increasing the aeration of the cultures. Within experimental limits, changing the culture volume per flask which would be expected to increase the aeration of the cultures had no effect. The results are shown overleaf. This result indicates that under our conditions, aeration was probably not the factor limiting the experimentally determined growth yield from reaching the value found by Kormancikova et al. (Kormancikova et al., 1969).

LEGEND TO FIGURE 7.8.

Growth Yield Analysis of D6 and D22

0.2% inocula of stationary phase cultures of D6 and D22 were added to a series of flasks containing 100 ml of YEP media and various concentrations of ethanol ranging from 1.7 to 8.5 mMoles. Growth was at 30°C. Other procedures were as described in chapter 2.

× D6

• D22

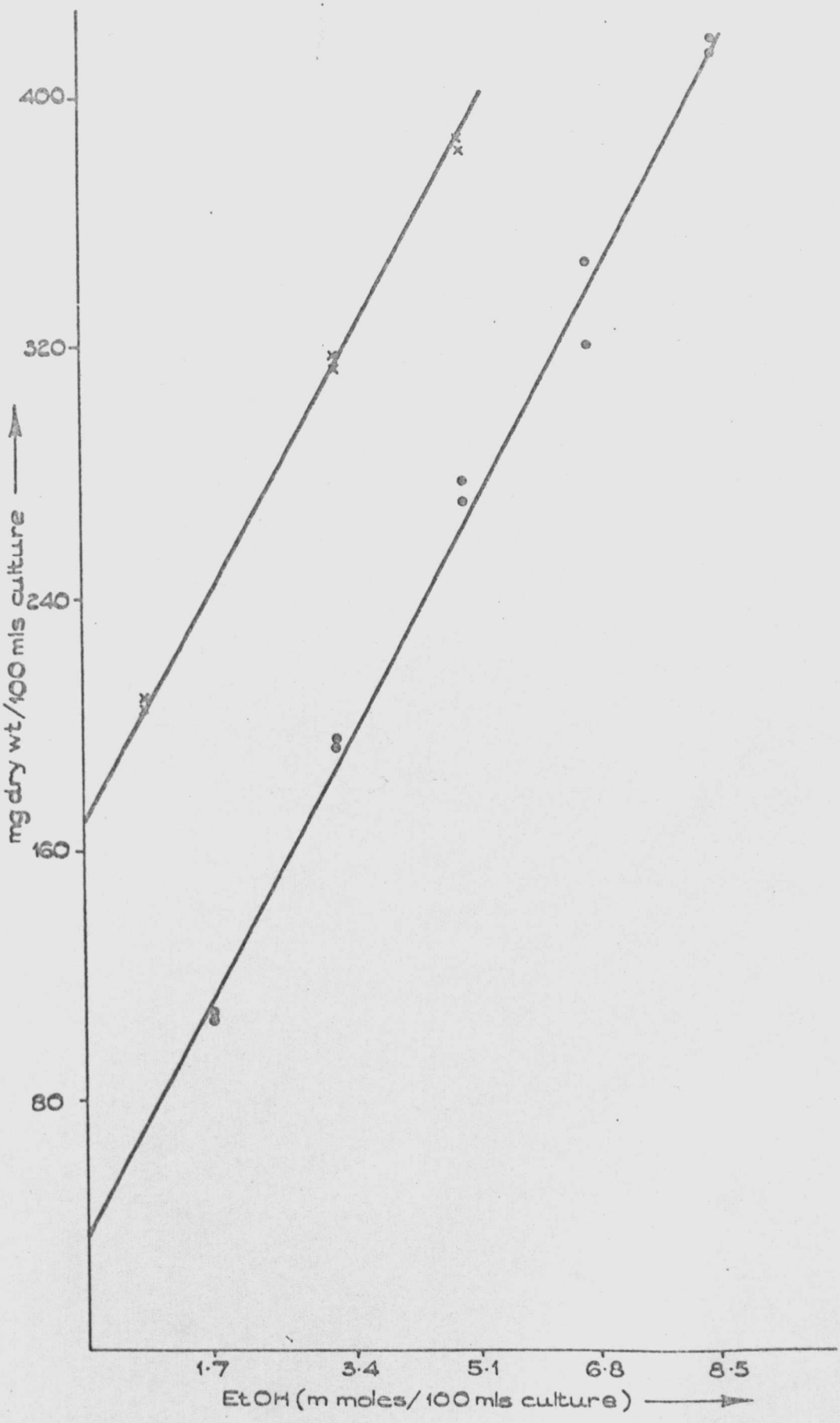

400
320
240
160
80
mg dry wt/100 mls culture
1·7
3·4
5·1
6·8
8·5
EtOH (m moles/100 mls culture)

TABLE 7.3.

Molar Growth Yields of OL^S and OL^R Strains

Strain	Class	Composition of the Growth Medium	Y_s	$\frac{Y_s \text{ Strain 'X'}}{Y_s \text{ D22 (1)}} \times 100$
D22 (1)		1% YE 2% Peptone	43.0, 42.0, 42.6 Mean = 42.5	100
D22		2% YE 4% Peptone	43.0	101.2
D22		Wickerhams Supplemented	46.0	108.3
D6		1% YE 2% Peptone	42.0, 41.4 Mean = 41.7	98.1
D22 A4	Ia	1% YE 2% Peptone	35.8	84.2
D22 A7	Ia	1% YE 2% Peptone	35.8, 37.0 Mean = 36.4	85.6
D22 A12	Ib	1% YE 2% Peptone	31.0, 33.0, 36.2 Mean = 33.4	78.6
D22 A15	IIb	1% YE 2% Peptone	42.0, 43.2 Mean = 42.6	100
D22 A16	IIb	1% YE 2% Peptone	42.6	100
D22 A21	IIb	1% YE 2% Peptone	42.6	100
D22 B3	I b	1% YE 2% Peptone	35.6	83.8
D22 B9	Ia	1% YE 2% Peptone	34.0, 36.0, 37.0, 39.1 Mean = 36.5	85.8
D22 B21	II a	1% YE 2% Peptone	42.5	100

Volume of Medium in 500 ml Culture Vessel	Y_s Exhibited by Cultures Measured at 2 EtOH Concentrations	
	6.5 moles EtOH	8.5 moles EtOH
25 ml	-	44.6
50 ml	40.8	44.5
100 ml	41.5	41.5

Effect of Changes in Culture Volume on the $Y_{substrate}$ Value Exhibited by Strain D22

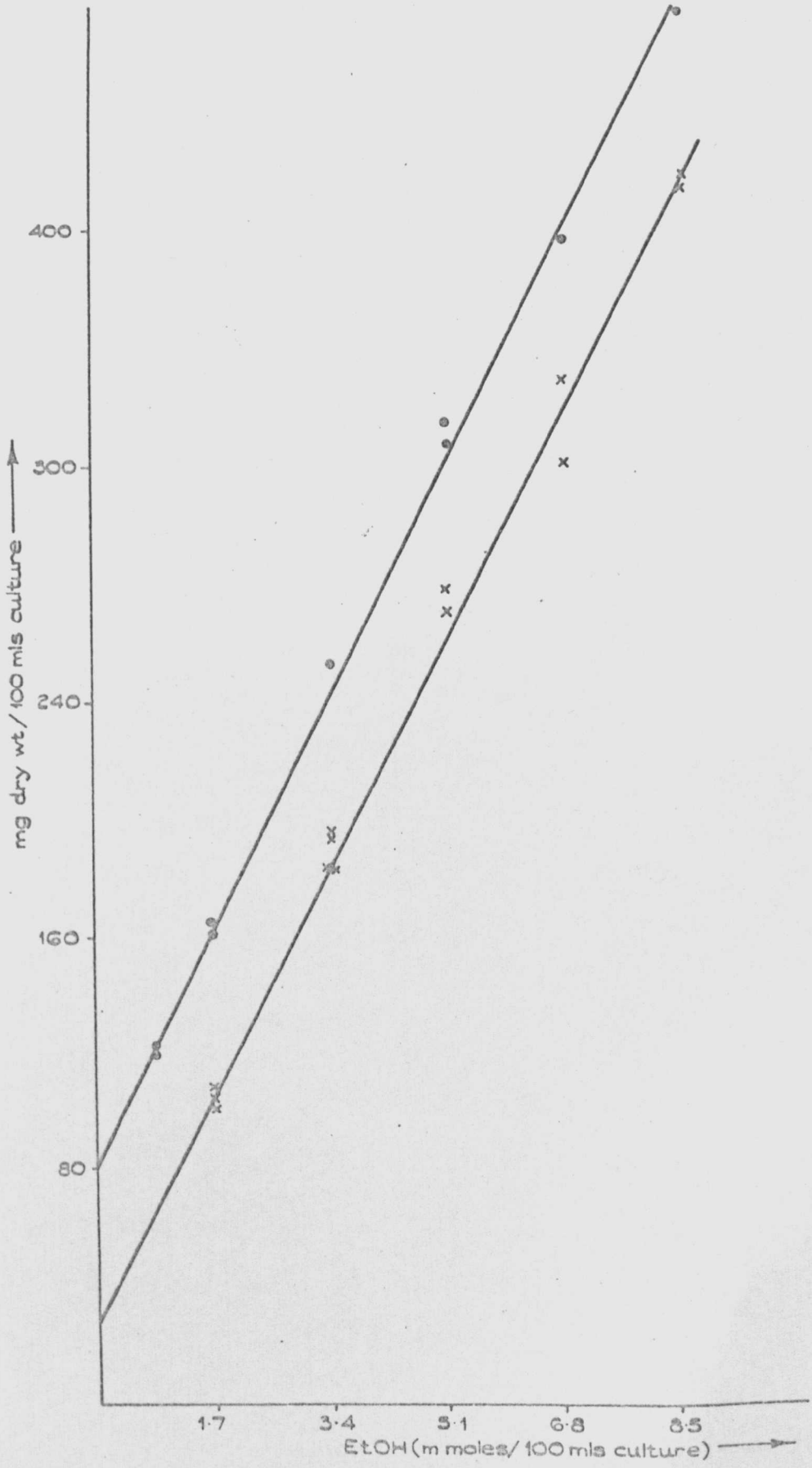
The possibility existed that the low $Y_{substrate}$ value was due to a deficiency in the medium leading to ethanol being catabolised to provide carbon skeletons for biosynthesis, this resulting in a reduced growth yield. Attempts to improve the observed $Y_{substrate}$ by using a richer basal medium (doubling the concentration of yeast extract and peptone used) did not, however, alter the $Y_{substrate}$ found though the growth of the strain in the absence of added ethanol, as expected, increased (Figure 7.9). The discrepancy between these results and those of Kormancikova et al. (Kormancikova et al., 1969) remain to be explained but may possibly be due to the use of wild type strains D6 and D22 which are auxotrophic for arginine and methionine and adenine respectively. Since the introduction of auxotrophic markers into a strain often results in its slower growth, this might be expected to lead to a decreased cell yield as the energy expended as maintenance energy will be proportionally greater. Alternatively, as in neither these experiments nor those of Kormancikova et al. has the amount of ethanol retained as cellular material been calculated, this may be the source of the discrepancy. In one sense it was expected that, if carbon was being retained as cellular material and not being fully oxidised, under enriched conditions (Figure 7.9) the growth yield would increase due to the sparing effects of the additional substrates on ethanol usage. This does, however, make certain assumptions as to the lack of cellular discrimination in favour of internally generated cellular building blocks. Moreover, there will be no sparing effect if the ethanol is being used solely to provide carbohydrate material as the enriched

LEGEND TO FIGURE 7.9.

Effect of Different Yeast Extract / Peptone Concentrations on the Growth Yield of D22

A 0.2% inoculum of a stationary phase culture of strain D22 was made into a series of flasks containing the two media detailed below and a graded series of ethanol concentrations as the carbon source. Growth was at 30°C and dry weight analysis was performed as described in chapter 2.

- • 4% Peptone, 2% Yeast Extract
- × 2% Peptone, 1% Yeast Extract


400
300
240
160
80
mg dry wt / 100 mls culture
1·7
3·4
5·1
6·8
8·5
EtOH (m moles/ 100 mls culture)

media will still require this to be provided almost totally from the ethanol carbon source.

Figures 7.10. and 7.11. and Table 7.3. show the results of a growth yield analysis of different OL^R strains derived from D22. Whilst none of the class II mutants tested had an altered growth yield (Figure 7.11), all the class I mutants tested, whether of sub-classes a or b, had their growth yield decreased by 15 - 20% (Figure 7.10). All the values listed in Table 7.3. were obtained in the manner shown for the graphically illustrated experiments, using a range of ethanol concentrations and establishing the linearity of the growth yield response to ethanol concentration (chapter 2). It therefore appears that the correlation and divisions already made on phenotypic and genotypic grounds between classes I and II extend to a fundamental, if small, difference in energy metabolism.

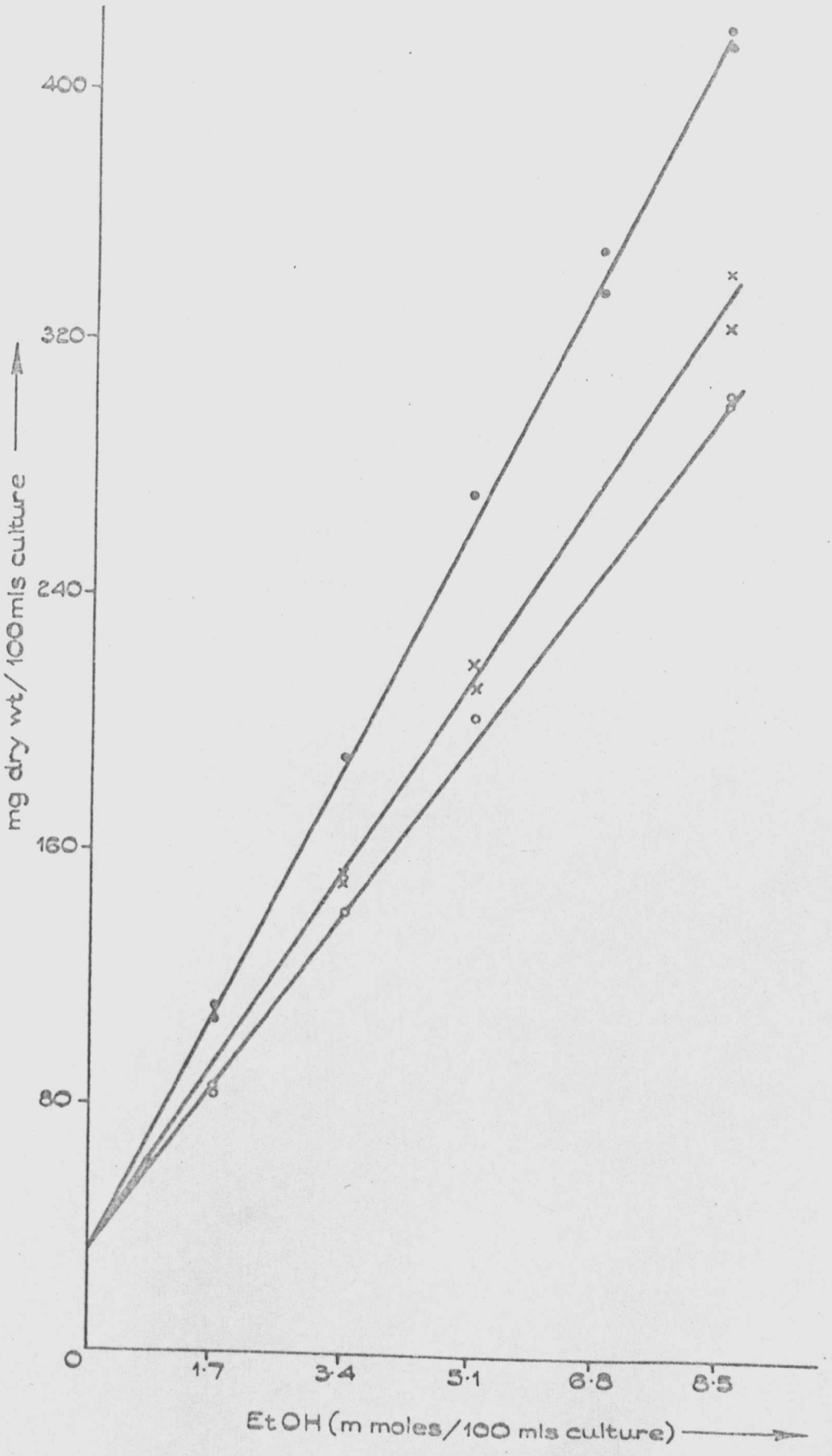
Experiments performed using certain types of sub-mitochondrial particles have shown that oligomycin at low sub-inhibitory concentrations actually stimulates the synthesis of ATP coupled to respiration as well as other reactions indicative of energy conservation such as the energy linked transhydrogenase reactions (Lee and Ernster, 1968; Haas, 1964; Kettman, 1965). By analogy to this effect of oligomycin which appears to be due to the prevention of the hydrolysis of high energy intermediates by the 'plugging' of the sub-mitochondrial particles, the effects of low sub-inhibitory concentrations of oligomycin on cell yield was examined. The results shown in Table 7.4. indicate that the presence of sub-inhibitory concentrations of oligomycin had little effect on the growth yield of either D6 or the D22 derived OL^R mutants of either class I or class II. The slight discrimination seen between the control and oligomycin treated cultures is within experimental error although, with the exception of D6, the oligomycin treated cultures did, in most cases, show fractionally higher mean growth yields. It therefore appears that in vivo either oligomycin does not have this effect or the effect is relatively unimportant to cell growth. Such a conclusion is not perhaps unexpected as even if the mitochondria in the intact cell are uncoupled or loosely coupled during various periods, the uncoupling may not be preventable by oligomycin, i.e. analogous to that produced by DNP etc. and not in any way similar to that seen in Mg^{2+} Edta mitochondrial particles (Lee and Ernster, 1968).

LEGEND TO FIGURE 7.10.

Growth Yield of D22 and D22 $OL^{R}1$ Strains

0.2% inocula of stationary phase cells of the various strains were added to a series of sidearm flasks containing graded amounts of ethanol in 100 ml of YEP medium. The concentrations of ethanol used varied from 1.7 to 8.5 mMoles.

- • D22
- o D22 A12
- × D22 B9


400
320
240
160
80
0
mg dry wt/ 100 mls culture
1·7
3·4
5·1
6·8
8·5
Et OH (m moles/100 mls culture)

LEGEND TO FIGURE 7.11.

Growth Yield of D22 and the D22 OL^{R}II Strains

The cultures were grown from a 0.2% inoculum of stationary phase cells on YEP media containing a graded series of ethanol concentrations as the carbon source. Growth was at 30°C, and dry weight estimations were carried out as described in chapter 2.

○ D22A21

● D22A16

× D22

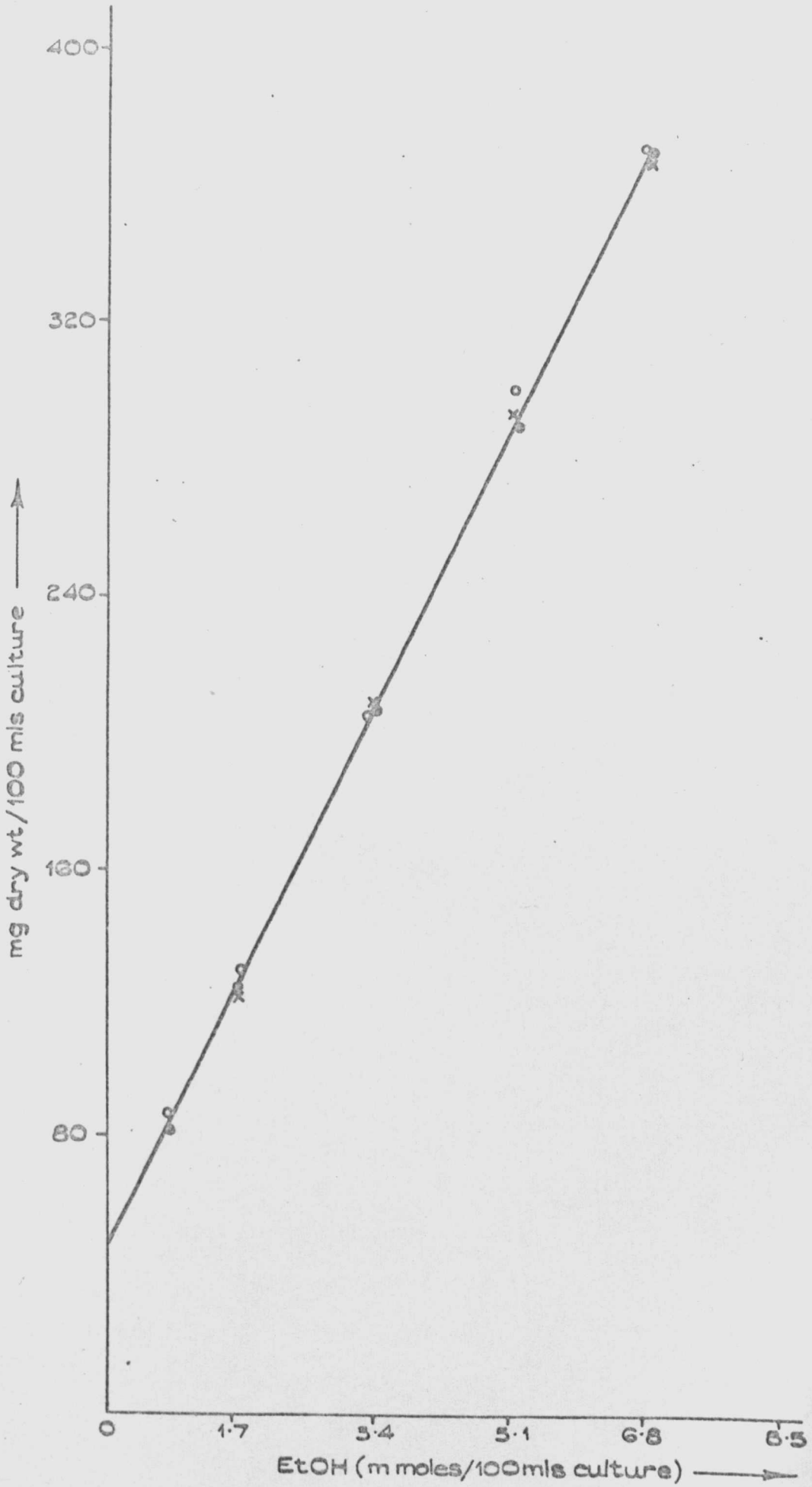

400
320
240
160
80
mg dry wt/100 mls culture
0
1·7
3·4
5·1
6·8
8·5
EtOH (m moles/100 mls culture)

TABLE 7.4.

Effect of Sub-Inhibitory Concentrations of Oligomycin on the Growth Yield of Various Strains

Strain	Oligomycin Concentration γ/ml	Dry Weight Yield gm/m mole Ethanol Total Ethanol Concentration moles 1.7	3.4	5.1	8.5
D6	-	-	-	49.1) 47.6) 48.3	45.5) 44.8) 45.2
D6	+ 0.25	-	-	43.1) 44.5) 43.8	43.3) 43.3
D22 A12	-	31.1) 29.7) 30.4	-	32.7) 33.3) 33.0) 33.0	31.3) 31.6) 31.5
D22 A12	+ 1.25	32.8) 36.0) 34.4		34.6) 33.6) 33.7) 32.9) 33.5	32.7) 31.5) 33.4) 33.6) 32.5
D22 B3	-	-	-	35.5) 35.5) 35.5	-
D22 B3	+ 1.25	-	-	38.0) 37.5) 37.8	-
D22 B9	-	-	34.2) 34.7) 34.4	38.5) 37.5) 38.0	34.0) 36.0) 35.0
D22 B9	+ 1.25	-	34.4) 36.7) 35.5	40.1) 40.7) 40.4	36.7) 35.6) 36.1

Electron microscopic examination of
D22 and D22 derived OL^R mutants

As the purpose of this investigation was to compare the ultrastructural properties and especially the mitochondrial characteristics of the wild type strain D22 and the oligomycin resistant mutants derived from it, it was imperative that all the strains be grown on non-fermentable media under closely similar or identical aeration conditions, and furthermore, be fixed in the mid-logarithmic phase of growth. Table 7.5. summarises the fixation procedures tried with intact cells in an attempt to fix them after growth under the conditions just described. In no case was reasonable fixation of cytoplasmic or nuclear elements achieved though older cells which were in early stationary phase were fixed with some degree of success using the procedure of Conti and Brooks (Table 7.5). As the fixation achieved with intact mid-log phase cells was so poor, the alternative procedure of removing the cell wall to allow penetration of the fixative by making spheroplasts was used. Such a procedure has, of course, its own disadvantages in that the osmotic lability of the spheroplasts can lead to distortion during the fixation process which involves the use of high osmolarity fixatives. The results of a series of fixation trials using either $MgSO_4$ or sorbitol as the osmotic stabiliser in both the preparation and fixation procedures, combined with a variety of fixatives are shown in Table 7.6. Sorbitol in our hands has consistently proved to be the more efficient osmotic stabilising agent giving preparations containing many more intact spheroplasts than $MgSO_4$. It was therefore routinely adopted as the osmotic stabiliser of choice. Of the fixation procedures tried, those using 5% glutaraldehyde were more successful than those using the lower concentrations of glutaraldehyde, but even this concentration was only effective when followed by OsO_4 postfixation - the use of permanganate as a postfixative leading to gross disorganisation of the spheroplasts. None of the procedures involving solely glutaraldehyde as the primary fixative has given really satisfactory fixation. For instance, cells fixed using glutaraldehyde alone often contain mitochondria which appear swollen and the cell profiles frequently showed crenellations characteristic of a sizeable loss of volume during fixation. The problem of fixing the spheroplasts has been overcome by the use of Karnofsky's fixative (Karnofsky, 1965), coupled with osmium tetroxide postfixation. This procedure has given cell profiles of good quality as

TABLE 7.5.

Fixation Trials on Intact Cells

This list is not exhaustive

Outline of Fixation Treatment Used	Reference	Quality of Fixation
2% glut. / buffered P ; 2% OsO_4 in Millonig's buffer ; 1% uranylacetate	(Heslot et al. 1970)	None
5% unbuffered $KMnO_4$; 1% uranyl-acetate / 1% potassium dichromate	(Avers et al. 1965)	None
1% unbuffered $KMnO_4$; 0.5% uranyl-acetate		
1.5% unbuffered $KMnO_4$; 2% OsO_4	(Conti and Brock 1965)	Fair
3% Acrolein and glut.	(Marchant and Smith 1967)	None
2% glut. in culture medium ; 2% $KMnO_4$; 1% uranylacetate / potassium dichromate	(Damsky et al. 1969)	None
2% $KMnO_4$ / veronal acetate buffer ; 2% uranylacetate	(Vitols et al. 1961)	None
1.5% unbuffered $KMnO_4$; 1% uranyl-acetate	(Smith et al. 1969)	None
2% formaldehyde ; RNAase treatment ; 1% OsO_4 / veronal acetate buffer	(Yotsuyanagi 1962)	None
2% OsO_4 / Millonig's buffer ; 0.5% uranylacetate	(Yotsuyanagi 1962)	None
2% $KMnO_4$ / veronal acetate buffer ; 1% uranylacetate / 1% potassium dichromate	(Linnane et al. 1962)	None
3% glut. / Cacodylate buffer ; 1% or 2% OsO_4 ; 0.5% uranylacetate	(Marchant and Smith 1967)	None

TABLE 7.6.

Fixation carried out unless otherwise stated at $0^{\circ}C$.

Cit P = Citrate phosphate buffer

P = Phosphate buffer

R.M. = Room temperature

TABLE 7.6.

Fixation Procedure Trials with Spheroplasts

Fixation Procedure	Comments
5% Glutaraldehyde / 0.04 M $MgSO_4$, Cit P - 4 hours 1% OsO_4 / P - 12 hours	Lysed
5% Glutaraldehyde / 0.1 M Sorbitol, Cit P - 4 hours 1% OsO_4 / P - 12 hours	* Mitochondria swollen
5% Glutaraldehyde / 0.04 M $MgSO_4$, Cit P - 4 hours 5% $KMnO_4$ - 1 hour at R.M.	Lysed
5% Glutaraldehyde / 0.1 M Sorbitol, Cit P - 4 hours 5% $KMnO_4$ - 1 hour at R.M.	* Mainly lysed
5% $KMnO_4$ - 75 minutes at R.M.	Lysed
2.5% Glutaraldehyde / 1% NaCl + 0.5% $CaCl_2$ - 4 hours 1% OsO_4 / P - 12 hours	** Mitochondria still swollen
3% Glutaraldehyde / 0.15 M Sorbitol, Cit P - 2 hours 1% OsO_4 / P - 12 hours	* Mitochondria swollen
3% Glutaraldehyde / 0.15 M Sorbitol, Cit P - 2 hours 3% $KMnO_4$ - 1 hour at R.M.	Lysed
5% Glutaraldehyde / 0.05 M, Cit P - 2 hours 1% OsO_4 / P - 12 hours	** Very heterogeneous sample
Karnofsky's Fixative - 2 hours 1% OsO_4 / P - 12 hours	***
Half-strength Karnofsky's - 2 hours 1% OsO_4 / P - 12 hours	***

KEY

* Poor Fixation → *** Good Fixation

has the use of half strength Karnofsky's fixative coupled with the osmium tetroxide postfixation procedure. Whilst cellular membranes appear rather more distinct after the former treatment, there are still some signs of shrinkage of the spheroplast at the cell edge. This effect is absent with the latter treatment, though the cellular membranes appear slightly less distinct.

Table 7.7. shows that for the majority of the strains analysed, electron micrographs were obtained using both the Karnofsky's fixative and the half-strength Karnofsky's fixative. Furthermore, the cells subjected to fixation were obtained in the case of six out of the eight analyses undertaken from more than one independent logarithmic phase culture. This analysis was concerned primarily with the presence or absence of morphological changes caused by the mutation conferring oligomycin resistance but the effect of sub inhibitory concentrations of oligomycin on the morphology of the resistant strains has also been examined.

Some of the electron micrographs obtained are shown in plates 7 - 12. The results of the analyses of the electron micrographs for the different strains grown in the presence and absence of oligomycin are shown in Table 7.8. and also in the accompanying histograms illustrated in Figures 7.12, 7.13 and 7.14. Although the number of cell profiles analysed is not great, in the case of D22, both this wild type and D22 A15 appear to have a slightly larger mean cell area than D22 A7, D22 A12 and D22 B9. The difference between the mean cell area of D22, D22 A15 and the three class I mutants is, however, maximal in the case of D22 A7, and even here amounts to only 30%. No systematic differences in the mean nuclear area or in the percentage area of the cell occupied by the nucleus are apparent between the strains and due to the comparatively small number of samples in which the nuclear membrane was clearly delineated throughout its entire length, the number of observations made is comparatively small (Table 7.8). The percentage area of the cell profiles occupied by the nucleus appears in four out of five of the strains when grown in the absence of oligomycin, to be between 12.5 and 14.7%. In the case of D22 A7 the figure obtained was higher, reaching 19.7%.

The capacity of the cell for making ATP might be expected to depend on a number of factors. These will include the number of mitochondria per cell, the cristal area per mitochondria, the activity of the electron transport assemblies per unit area of the cristal membrane and the efficiency of production of 'high energy'

TABLE 7.7.

Fixation Procedures Used with Spheroplasted Yeast Strains

Strain	No. of Individual Cultures Treated	Fixation Procedures Used
D22	2	5% Glut. in 0.1 M Sorbitol / Citrate P buffer - 1% OsO_4 Karnofsky's - 1% OsO_4
D22 A7	2	Karnofsky's - 1% OsO_4 ½ strength Karnofsky's - 1% OsO_4
D22 A7 + OL	1	½ strength Karnofsky's - 1% OsO_4
D22 A12	2	Karnofsky's - 1% OsO_4 ½ strength Karnofsky's - 1% OsO_4
D22 A12 + OL		Karnofsky's - 1% OsO_4 ½ strength Karnofsky's - 1% OsO_4
D22 A15	2	Karnofsky's - 1% OsO_4 ½ strength Karnofsky's - 1% OsO_4
D22 A15 + OL	1	½ strength Karnofsky's - 1% OsO_4
D22 B9	2	Karnofsky's - 1% OsO_4 ½ strength Karnofsky's - 1% OsO_4

TABLE 7.8.

Analysis of Electron Micrographs

Parameter	Strain							
	D22	D22A7	D22A7*	D22A12	D22A12*	D22A15	D22A15*	D22B9
Cell Profile Area um^2	8.8 (18)	6.0 (36)	5.2 (18)	6.1 (14)	5.1 (22)	8.4 (23)	5.2 (13)	7.4 (34)
Nuclear Area um^2 / Cell Profile	1.2	1.2	1.0	0.9	1.2	1.3	0.9	0.9
Percentage Nuclear Area / Cell Profile	13.5	19.0	18.2	14.7	22.0	15.0	16.5	12.4
Number of Mitochondria / Cell Profile	7.6	8.0	7.1	9.8	6.7	9.9	9.0	8.5
Mean Area of Individual Mitochondria um^2	0.073	0.078	0.079	0.080	0.092	0.061	0.055	0.079
Total Mitochondrial Area um^2 / Cell Profile	0.73	0.62	0.53	0.75	0.59	0.62	0.46	0.68
Percentage Mitochondrial Area / Cell Profile	8.3	10.3	10.2	12.3	11.6	7.4	9.0	9.2
Number of Mitochondria / Cell Profile (calculated)	26.5	24.9	22.1	27.9	19.7	36.5	30.0	28.2

* Culture + Oligomycin

Numbers in brackets = number of samples analysed

LEGEND FOR PLATES 7 - 12

D22 x45,000

D22 A7 x 40,000

D22 A12 x 43,000

D22 A12 + oligomycin x 45,000

D22 A15 x 35,000

D22 B9 x 53,000

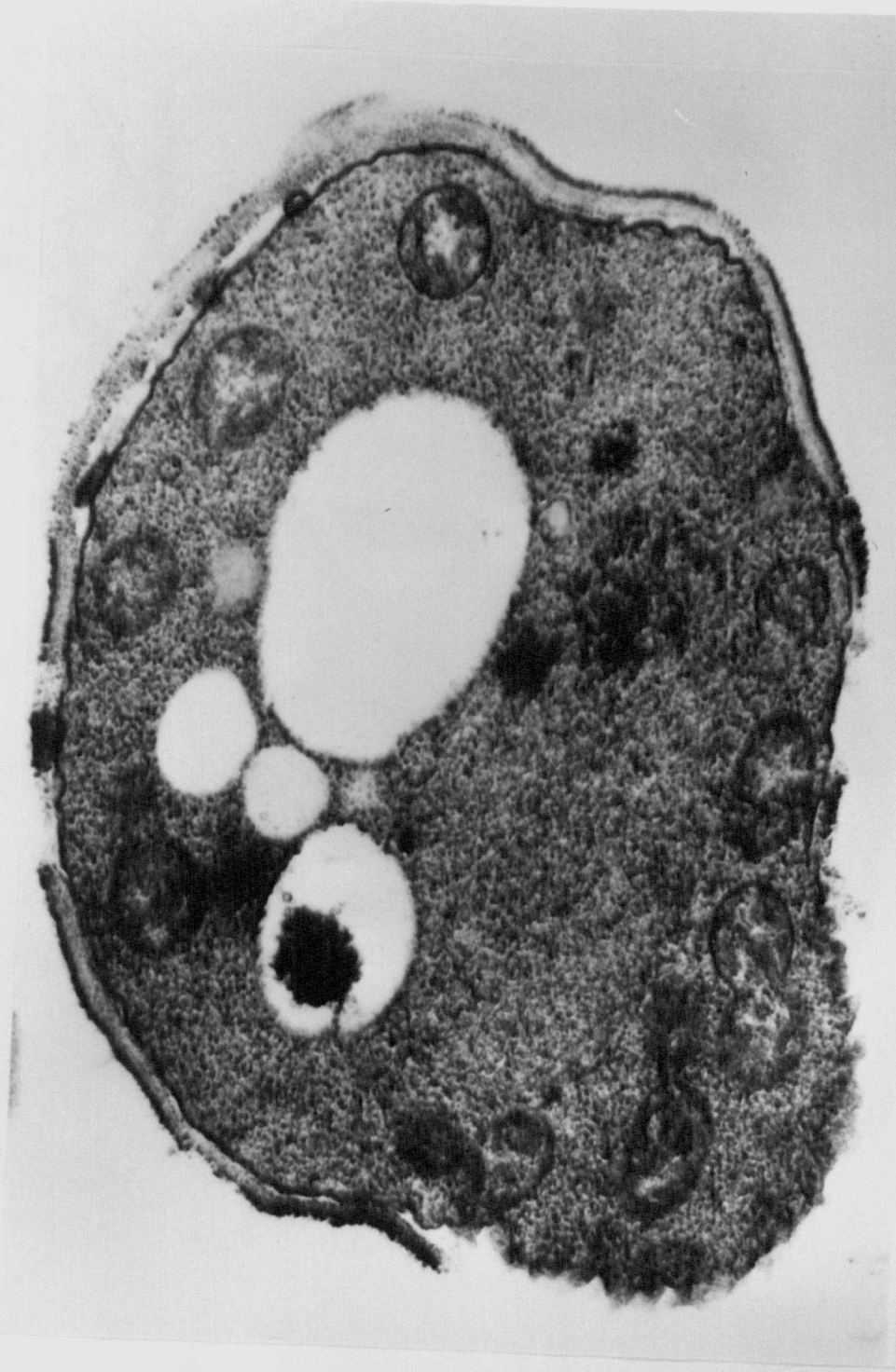


PLATE 7 D22

PLATE 8 D22 A7

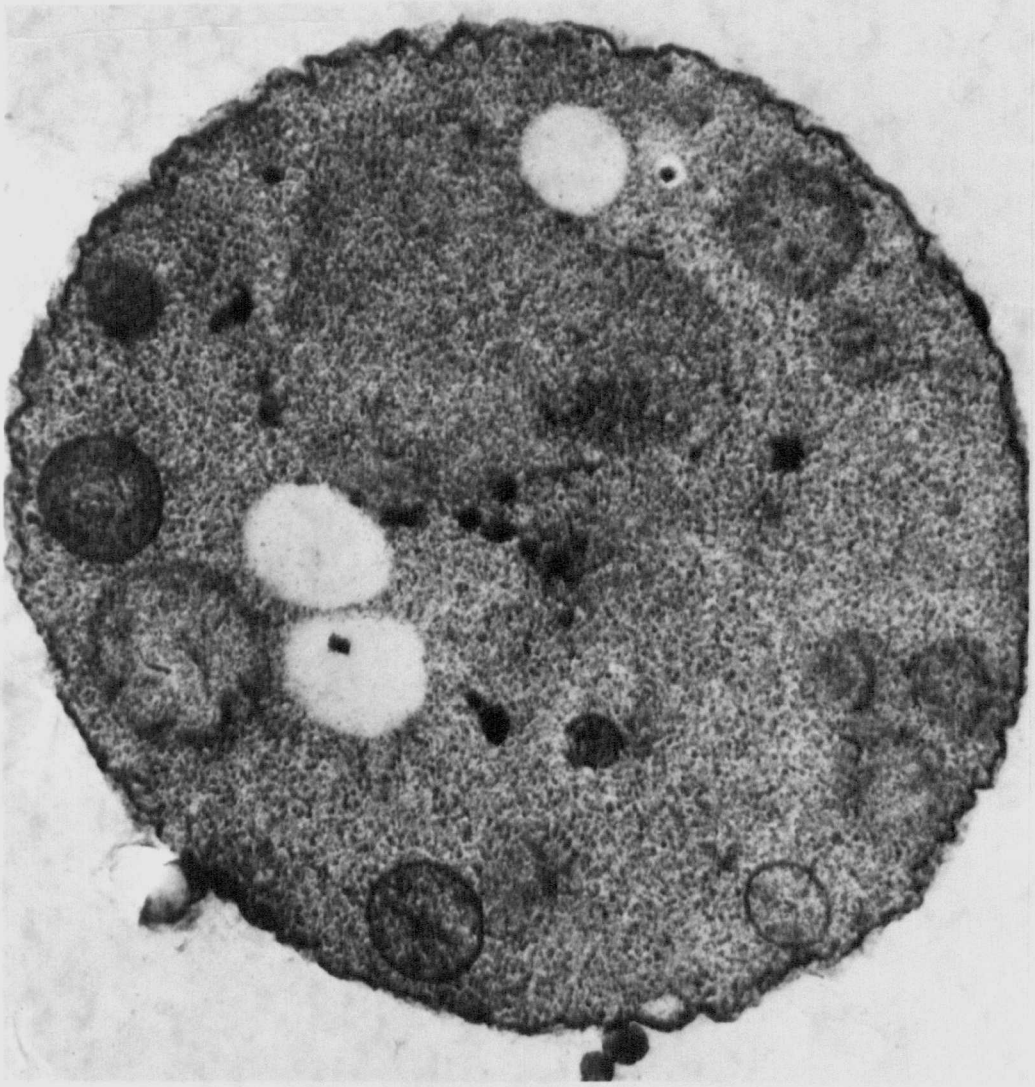


PLATE 10 D22 A12 + Oligomycin

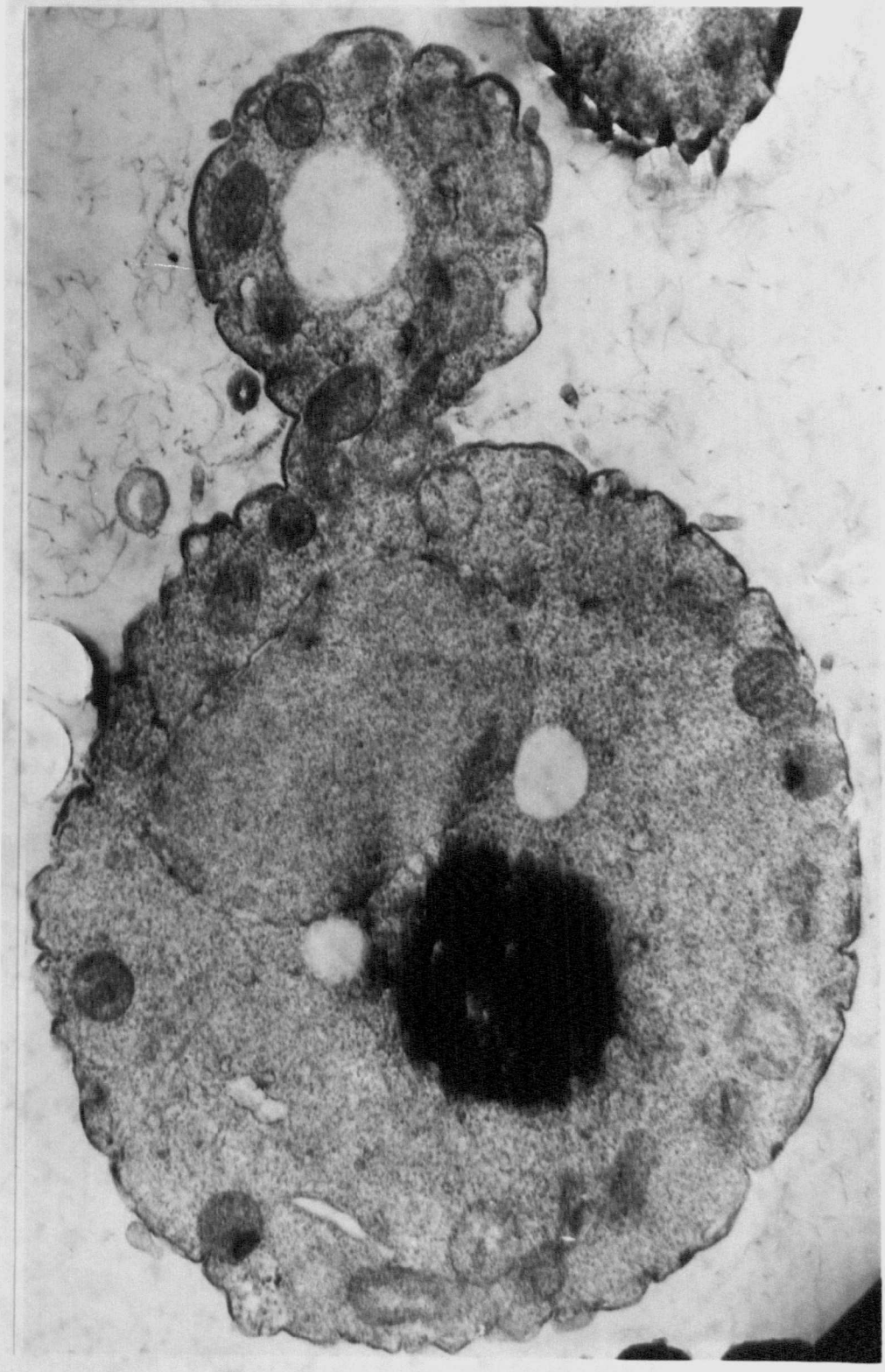


PLATE 11 D22 A15

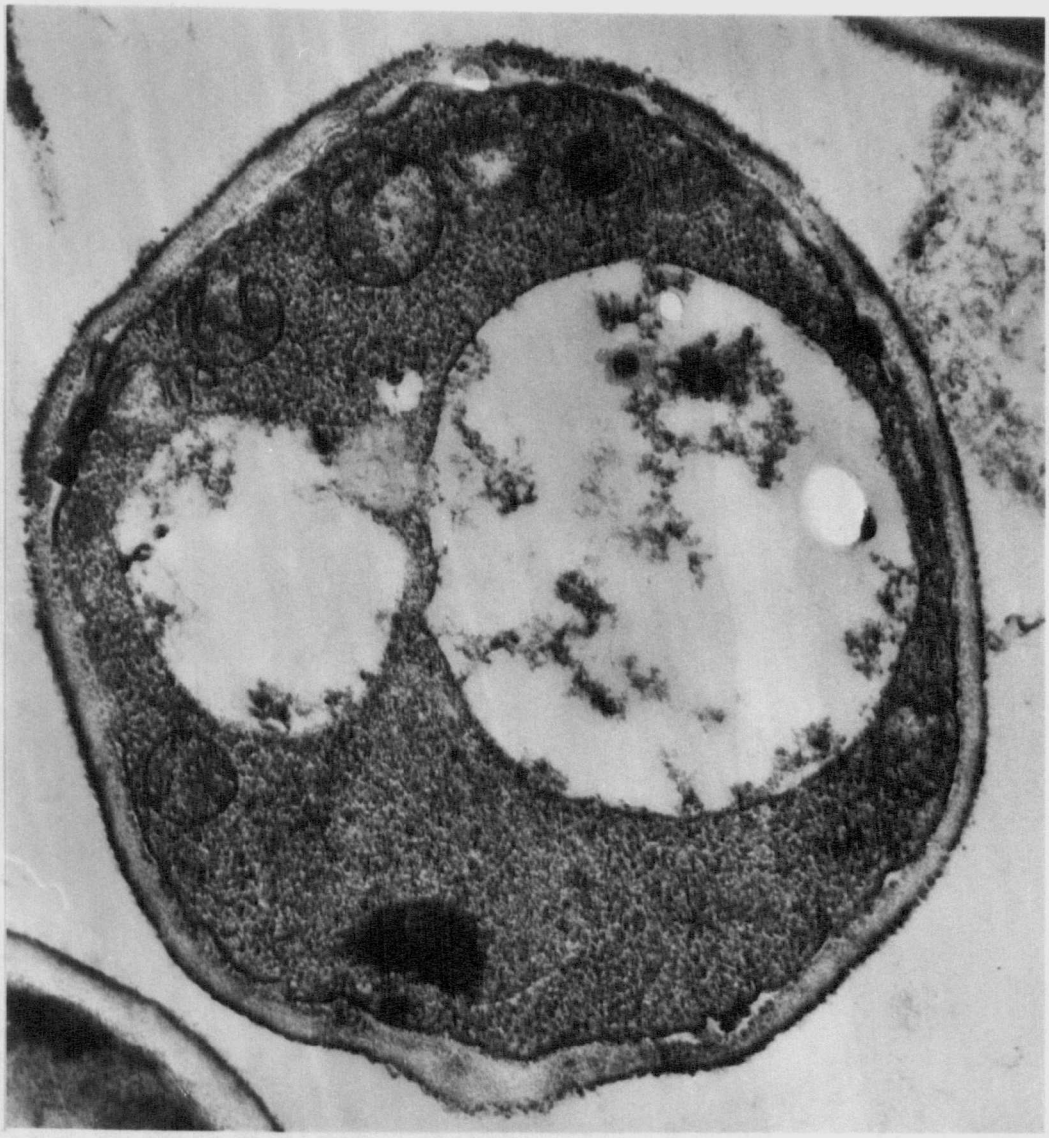
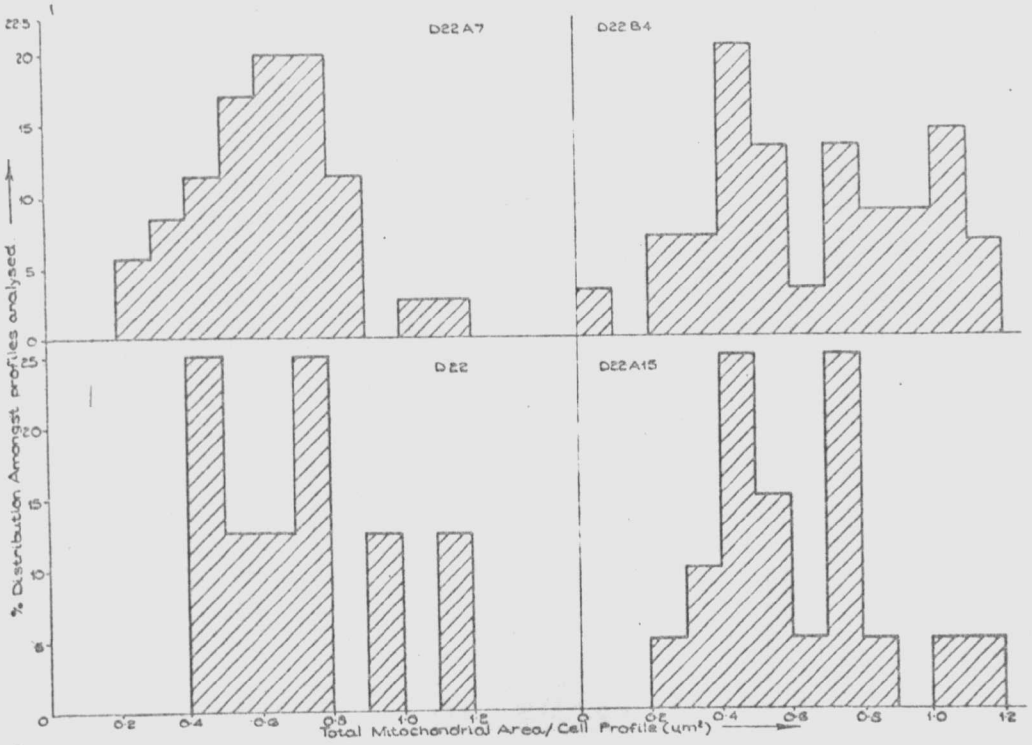
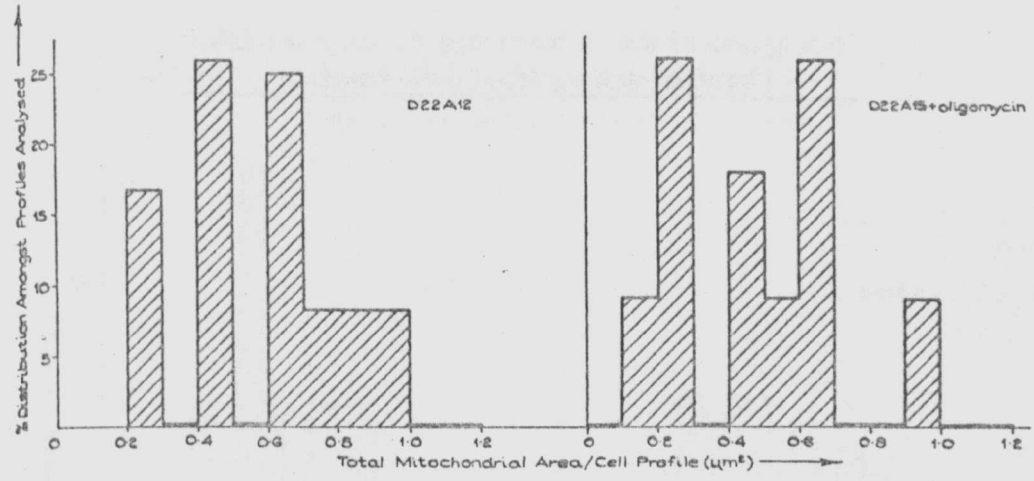


PLATE 12 D22 B9

FIGURE 7.12.

Histogram of the Distribution of Total
Mitochondrial Area in Individual Cell Profiles


D22A7
D22B4
D22
D22A15
% Distribution Amongst profiles analysed
Total Mitochondrial Area/Cell Profile (μm²)


D22A12
D22A15+oligomycin
% Distribution Amongst Profiles Analysed
Total Mitochondrial Area/Cell Profile (μm²)

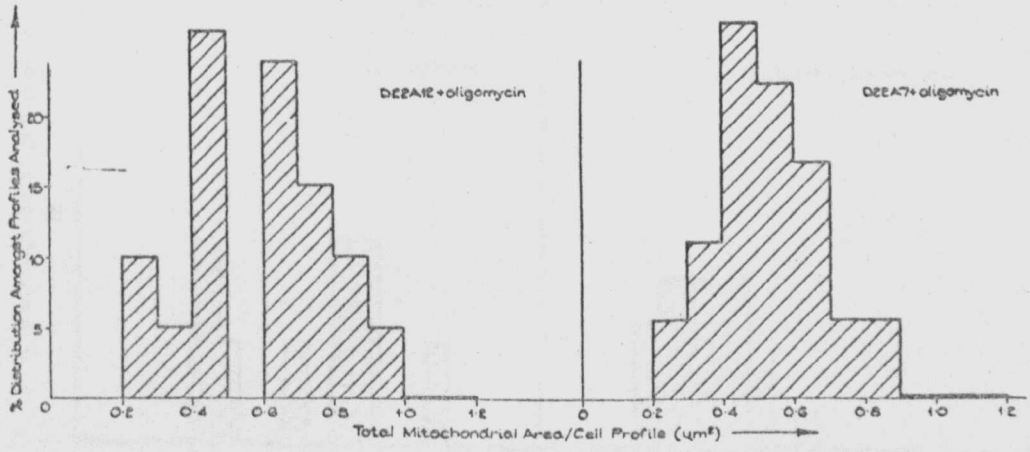

D22A12+oligomycin
D22A7+oligomycin
% Distribution Amongst Profiles Analysed
Total Mitochondrial Area/Cell Profile (μm²)

FIGURE 7.13.

Histogram of the Distribution of Mitochondrial Number in Individual Cell Profiles

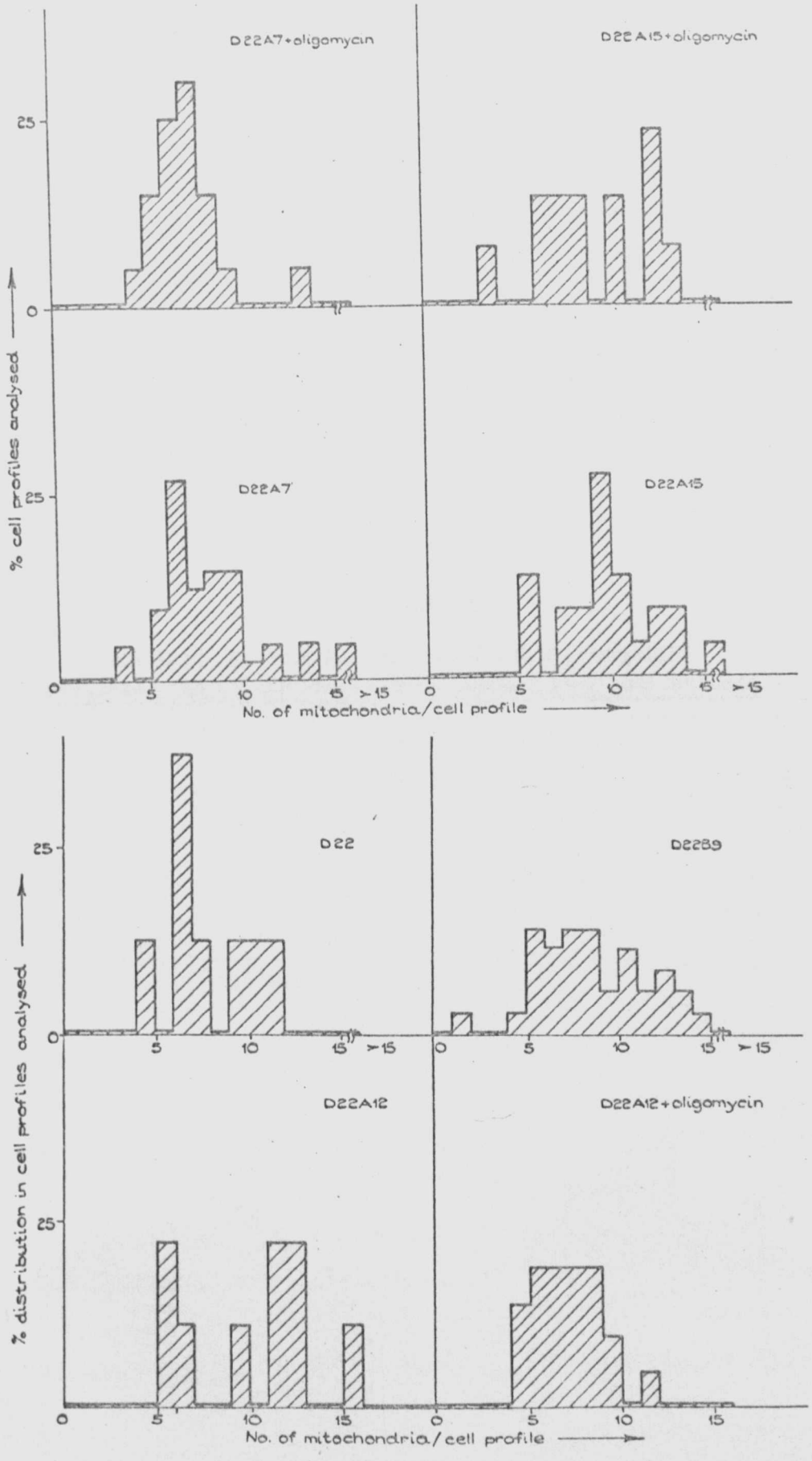
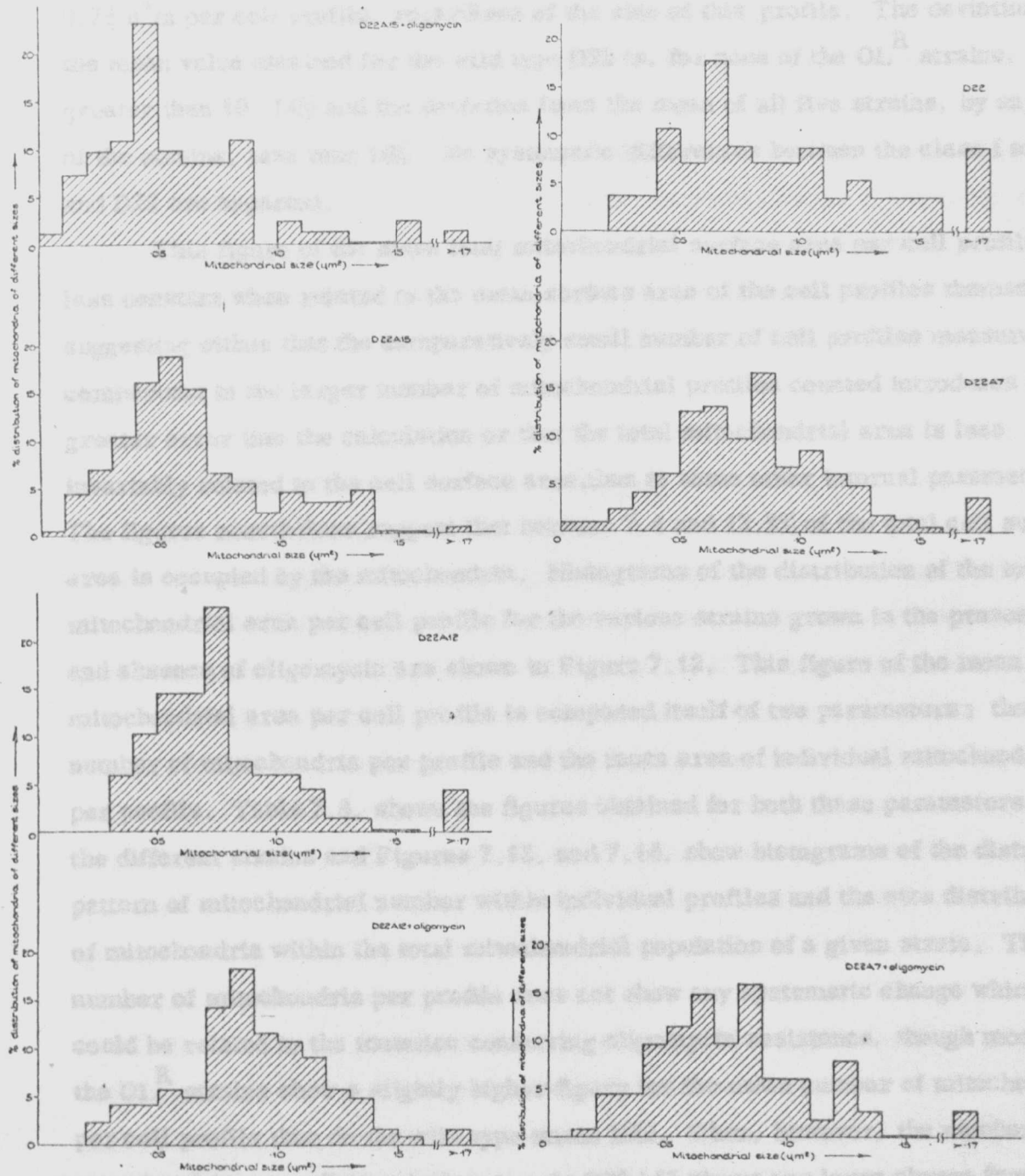

D22A7+oligomycin
D22A15+oligomycin
D22A7
D22A15
% cell profiles analysed
No. of mitochondria/cell profile
D22
D2289
D22A12
D22A12+oligomycin
% distribution in cell profiles analysed
No. of mitochondria/cell profile

FIGURE 7.14.

Histogram of the Distribution of Mitochondrial Size
Amongst the Total Number of Mitochondrial Profiles Analysed


D22A15+oligomycin
D22
D22A15
D22A7
D22A12
D22A12+oligomycin
D22A7+oligomycin
% distribution of mitochondria of different sizes
Mitochondrial size (μm²)
0·5
1·0
1·5
>1·7

compounds such as ATP. Although it is not possible to estimate the total cristal or inner membrane surface per cell or mitochondrion, an attempt has been made, using the electron micrographs, to estimate both the number of mitochondria and their surface area in the cell profiles of the various strains examined. The mean total mitochondrial surface area of the cell profiles of the various strains grown in the absence of oligomycin is virtually invariable, amounting to between 0.62 and 0.73 u^2m per cell profile, regardless of the size of this profile. The deviation from the mean value obtained for the wild type D22 is, for none of the OL^R strains, greater than 10 14% and the deviation from the mean of all five strains, by any one of the strains, less than 10%. No systematic differences between the class I mutants and D22 are apparent.

This figure of the mean total mitochondrial surface area per cell profile is less constant when related to the mean surface area of the cell profiles themselves, suggesting either that the comparatively small number of cell profiles measured in comparison to the larger number of mitochondrial profiles counted introduces a greater error into the calculation or that the total mitochondrial area is less invariably related to the cell surface area,than to some other internal parameter. The figures nonetheless suggest that between 7.4 and 12.3% of the total cell surface area is occupied by the mitochondria. Histograms of the distribution of the total mitochondrial area per cell profile for the various strains grown in the presence and absence of oligomycin are shown in Figure 7.12. This figure of the mean total mitochondrial area per cell profile is composed itself of two parameters ; the mean number of mitochondria per profile and the mean area of individual mitochondrion per profile. Table 7.8. shows the figures obtained for both these parameters in the different strains and Figures 7.13. and 7.14. show histograms of the distribution pattern of mitochondrial number within individual profiles and the size distribution of mitochondria within the total mitochondrial population of a given strain. The number of mitochondria per profile does not show any systematic change which could be related to the mutation conferring oligomycin resistance, though most of the OL^R strains show a slightly higher figure for the mean number of mitochondria per cell profile than do the wild type strain D22. When, however, the number of mitochondria per cell is calculated, only D22A15 shows any large change from the value exhibited by D22. This calculation is based on a model assuming the

spheroidicity of both the mitochondrion and the cell itself. Both these assumptions appear to be correct in the case of all but an insignificant number of the profiles examined. The mean value of the mitochondrial number per cell for the five strains grown in the absence of oligomycin is slightly high in comparison to the value of 40 - 50 which Avers et al. (Avers et al., 1965) have found for a diploid wild type strain. However, in view of the doubt surrounding whether a diploid cell has exactly double the mitochondrial component of a haploid, differences between strains and the use of stationary phase glucose grown cells by the latter workers, the results seem in good accord. Whilst D22 A15 of all the strains examined seems to have the largest number of mitochondria per cell, it also has individual mitochondria with the smallest mean area. The histogram of the size distribution of individual mitochondria suggests also that there is a shift towards smaller mitochondrial size values in this strain. The other four strains, in the absence of oligomycin, show virtually identical values for the mean area of individual mitochondria of around 0.073 - 0.080 $u^2 m$.

Most of the mitochondria seen in this analysis were spherical in marked contrast to the results obtained by Cottrell et al. with cells growing experimentally in continuous culture though with 3% glucose as the carbon source (Cottrell and Avers, 1971). Few aberrant or irregular mitochondria were seen and the mitochondria themselves appeared to be generally distributed at the periphery or close to the periphery of the cell profiles. The majority of the mitochondria examined were highly electron dense and cristal structure was often not prominent. There did not appear to be, however, any correlation between the ease of visibility of cristal structure and particular strains or between particular strains and types of cristal structure. Those preparations of spheroplasts which were less intact and showed some mitochondrial swelling, however, exhibited cristal structure with greater facility than the preparations used in this analysis which appear to have retained complete structural integrity.

Some of the profiles examined were marked by a regular, almost paracrystalline inclusion consisting of apparently stacked membranes. Again, this inclusion was not apparently associated with any particular strain. The physiological function of such membrane stacks is not known. The effect of oligomycin on all three OL^R strains which were grown in its presence (5.0 γ/ml) seems to depress cell size to some extent and also mitochondrial number, though not apparently necessarily the mean area of individual mitochondria. The overall effect is,

however, to reduce the mean value of the total mitochondrial area whilst leaving unchanged, or changed to a lesser extent, the total mitochondrial area expressed as a percentage of the total cell profile area. The finding of closely similar values for the number of mitochondria per cell in these treated cell analyses suggests the correctness of the aforementioned data. These results regarding the oligomycin treated cells suggest that oligomycin may be able to influence the cellular morphology of even oligomycin resistant mutants albeit to a minor degree.

Discussion

The finding that the oligomycin resistance of the class I and class II mutants is expressed immediately, together with the results of the DCCD analysis, provides good evidence that under normal growth conditions the resitant phenotypes are constitutively expressed. The observation in the majority of the mutants, whether of class I or class II, that the growth rate is unaltered, suggests either than in those strains where there is a difference, the mutation conferring oligomycin resistance is different from that in the other strains examined or that these strains have accumulated a secondary mutation affecting the growth rate. It seems, however, that the mutation to oligomycin resistance in both clases of mutants normally occurs without affecting the growth rate. In contrast to this result, the growth yield experiments have given results pointing again to the systematic difference between the class I and class II mutants. Although there is no definite evidence to show whether the change in growth yield is due to a difference in the incorporation of carbon skeletons into the cell, a difference in the efficiency of the energy conservation process itself or a difference in the growth yield per 'high energy conservation unit' conserved, given that oligomycin acts apparently exclusively at the mitochondrial level (see chapter 5) (Shaw, 1967), it appears reasonable to regard this evidence and the DCCD results as indicating strongly that the class I type of resistance is expressed at the mitochondrial level.

As the OL^R mutants were isolated from glycerol drug plates and are therefore capable of utilising non-fermentable carbon sources, it was never visualised that the mitochondria of these strains would be disorganised in any degree approximating that found in petites (Avers et al., 1965; Yotsuyanagi, 1962). Even in the petite strains the degree of disorganisation may not be all that large

(Smith et al., 1969), many of the electron microscopy studies being highly misleading in that the petites have been grown on medium often containing high concentrations of glucose so that the morphology due to the petite cell itself and of that due to glucose repression have been, to some extent, confused (Bowers, McClary and Ogur, 1967). The results of the electron microscopic analysis con firm this assessment, there being no evidence of systematic change in the mitochondrial ultrastructural parameters of either the class I or class II mutants. The significance of the finding that the number of mitochondria per cell is slightly higher in D22 A15 than in the other strains tested is uncertain and examination of further class II mutants, especially from the two recombination groups 'R' and 'S' is necessary before this is assigned as a characteristic of the OL^{R} II mutants. However, if this observation is found to be a characteristic of only some of the class II mutants, it may be of importance in reconciling the results of the allelism crosses with those of the three-point mapping where a certain lack of rapport is evident (chapter 6). It is, for instance, possible to imagine models where the polarity of recombination characteristic of a heterosexual cross, is contributed to either by all or only one of the following processes. Firstly, polarity of entrance of mDNA ; secondly, polarity of mitochondrial transfer from one strain to another and thirdly, polarity within the recombination event itself. Both the first and second processes may well be sensitive to the number of mitochondria per cell, assuming, at least as far as the first process is concerned, that the amount of mDNA per mitochondria is constant. Under these conditions if either the first or second process do contribute to polarity, then the number of mitochondria per cell may alter the mapping distances as measured by recombination frequencies.

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