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A COMPARATIVE STUDY OF MITOCHONDRIAL DIFFERENTIATION IN *CANDIDA PARAPSILOSIS* AND *SACCHAROMYCES CEREVISIAE*

by

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This thesis embodies results of research work carried out in the Department of Developmental Biology, Research School of Biological Sciences, Australian National University, during the tenure of an Australian National University Research Scholarship, for which I am indebted to the Council of the University.

Statement

Results described in this thesis were obtained by myself under the joint supervision of Dr. P.R. Stewart and Professor D.J. Carr. Part of the results presented in Chapters 4 and 5 of this thesis was obtained in collaboration with Dr. R. Poulson.

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Dedication

This thesis is my humble dedication to Professor D.J. Carr, whom I honour and respect.

Richard S-T Yu

Abbreviations

AMP	Adenosine Monophosphate (or Adenylicacid)
ADP	Adenosine Diphosphate
ATP	Adenosine Triphosphate
CAP	Chloramphenicol
CHI	Cycloheximide
CMP	Cytidylic Acid
CPSS	Cytoplasmic Protein Synthesising System
DNA	Deoxyribonucleic Acid
EB	Ethidium Bromide
EDTA	Ethylenediamine tetra acetic acid
ER	Erythromycin
GMP	Guanylic Acid
LP	Large Particles
MPSS	Mitochondrial Protein Synthesising System
MT	Mitochondria
PEP	Phosphoenolpyruvate
PMS	Postmitochondrial Supernatant
RNA	Ribonucleic Acid
rRNA	Ribosomal Ribonucleic Acid
sRNA,tRNA	Soluble or Transfer Ribonucleic Acid
SOL	Soluble fraction
TCA cycle	Tricarboxylic Acid cycle
TCA	Trichloroacetic acid
UFA	Unsaturated Fatty Acids
UMP	Uridylic Acid

SUMMARY

This thesis presents an attempt to determine if observations made on *S. cerevisiae* can be generally extrapolated to other organisms, such as *C. parapsilosis*. Comparative studies on the respiratory physiology and macromolecules of mitochondria of *C. parapsilosis* and *S. cerevisiae* relevant to mitochondrial differentiation were carried out. Main findings are:

(1) Classical environmental factors (catabolite repression and anaerobiosis) and genetic factors (mutagen-induced) controlling mitochondrial differentiation in *S. cerevisiae* do not exist in *C. parapsilosis*.

(2) *C. parapsilosis*, unlike *S. cerevisiae* which possesses only a cyanide-sensitive respiratory pathway, has two respiratory pathways, one being sensitive and the other insensitive to cyanide and Antimycin A. The insensitive pathway is specifically induced by $(NH_4)_2SO_4$ and metabolites of glucose.

(3) Treatment of *S. cerevisiae* with CAP or ER results in respiratory-deficiency lacking cytochromes aa_3 and b. Inhibition of synthesis of cytochromes aa_3 and b occurs in *C. parapsilosis*, but it depends on the growth phase of the cell. CAP-treated cells of *C. parapsilosis* have a normal level of respiration which is however insensitive to cyanide and Antimycin A. Synthesis of cytochrome c is not inhibited by CAP or ER in *S. cerevisiae* and *C. parapsilosis*. Also, formation of mitochondrial membranes and the MPSS does not depend on the activity of the MPSS in both *C. parapsilosis* and *S. cerevisiae*.

(4) The phasic response on the inhibition of synthesis of cytochromes aa_3 and b by CAP or ER in *C. parapsilosis*, the cause of which is related to the rate of cell growth, is a cellular regulatory mechanism by nature controlling synthesis of these cytochromes.

(5) Overall mitochondrial protein synthesis of *C. parapsilosis* and *S. cerevisiae* is similar in terms of cofactor requirements and antibiotic responses. However, mitochondrial

ribosomes of *C. parapsilosis* are 70*s* and those of *S. cerevisiae* 80*s*. RNA constituents of mitochondrial ribosomes of these organisms are different in molecular size.

(6) Mitochondrial RNA of these two organisms are similar in reactivity to solution environments, both showing similar thermally-induced conformational changes.

(7) Treatment of *S. cerevisiae* and *C. parapsilosis* with EB results in the inhibition of synthesis of mitochondrial ribosomal RNA and cytochromes aa_3 and *b*. The inhibition is permanent in *S. cerevisiae* but reversible in *C. parapsilosis*.

In conclusion, the control of mitochondrial differentiation is different in *C. parapsilosis* and *S. cerevisiae*. Respiratory physiology of these two organisms is different. Similarities of overall activities of the MPSS in these two organisms do not extend to the participating ribosomes and RNA constituents of these ribosomes.

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CHAPTER 1

GENERAL INTRODUCTION

CHAPTER I

1-1 COMPLEXITY OF FUNCTIONS IN MITOCHONDRIA

Overall aspects of mitochondrial function may be dissected into five main systems. The mitochondrion contains (1) a chain of enzymes for the transfer of electrons; (2) a system for generating electron donors for the chain, such as the TCA cycle and pathways for the oxidative degradation of fatty acids; (3) a system for the synthesis of ATP by the condensation of ADP and inorganic phosphate; (4) ancillary metabolic systems; and (5) a system associated with the development or differentiation and inheritance of the organelle. The first three systems constitute the classical mitochondrial function of respiration and phosphorylation. The ancillary metabolic systems include pathways for fatty acid biosynthesis (Smoly *et al.*, 1970), synthesis of some amino acids (Berquist *et al.*, 1969), lipid synthesis and catabolism, the urea cycle and glutamate and aspartate biosynthesis (Mahler and Cordes, 1966) as well as several oxidative enzymes not directly involved with oxidative phosphorylation, for example a rotenone-insensitive NADH: cytochrome *c* reductase, monoamine oxidase and kynurenine hydroxylase (Smoly *et al.*, 1970). The system in the mitochondrion which is concerned with its differentiation and with its inheritance involves protein synthesis, transcription of genetic information encoded in mitochondrial DNA and replication of mitochondrial DNA.

These multienzyme systems in mitochondria are well-documented and extensively discussed in textbooks and reviews, and it is beyond the scope of this thesis to consider them further (for example see Lehninger, 1964; Green and Baum, 1970; Racker, 1970; Schatz, 1970; Ashwell and Work, 1970; Kuntzel, 1971).

1-2 ADVANTAGES OFFERED BY *S. CEREVISIAE* IN STUDIES OF MITOCHONDRIAL DIFFERENTIATION

The importance of mitochondria to cellular function

cannot be over-emphasized, and hence studies of the synthesis and regulation of multienzyme complexes in mitochondria are of the utmost interest. It is clear from a perusal of the literature on mitochondrial biogenesis or differentiation that perhaps an undue degree of attention has been given to the facultative anaerobe, *Saccharomyces cerevisiae*. The reasons for this are clear in that this organism offers two convenient experimental systems, namely respiratory adaptation and glucose derepression. These two experimental systems are ideal because during anaerobiosis or glucose repression concentrations of respiratory enzymes are nil or minimal. These two developmental processes present controlled experimental systems in which to study the regulation of *de novo* synthesis of respiratory enzymes. Studies using these two systems have been greatly facilitated by the discovery of specific inhibitors of protein, and recently of nucleic acid synthesis. It has become customary to use chloramphenicol (or erythromycin) and cycloheximide in these studies because they provide a relatively straightforward approach to questions of the site of synthesis and source of genetic information for mitochondrial components. Furthermore, this organism provides a convenient tool for analysis of the origin and regulation of synthesis of mitochondrial components, viz. by genetic analysis. It is perhaps worthwhile considering in more detail the external and intrinsic factors regulating mitochondrial differentiation and development in *Saccharomyces*.

1-2-1 ANAEROBIOSIS (CONTROL BY OXYGEN)

Anaerobically grown cells of *S. cerevisiae* contain well-defined mitochondria with double membranes and cristae (Wallace, Huang and Linnane, 1968; Yu, 1968), but lack respiratory activity due to the absence of the electron transfer chain (Wallace and Linnane, 1964). Enzymes of the TCA cycle, ATPase, flavin and ferrochelatase are apparently synthesized in near normal amounts (Schatz, 1970). Lipid and protein content of mitochondria are not radically different (Schatz, 1970) and thus physical properties of the organelle such as buoyant density are

unaffected.

Also, mitochondria isolated from anaerobically-grown cells contain DNA which has the same buoyant density as that of aerobic mitochondria (Schatz, 1970). Furthermore *in vitro* (Davey, Yu and Linnane, 1969) and *in vivo* (Schatz and Saltzgaber, 1969) amino acid incorporation experiments have demonstrated that anaerobically grown cells of *S. cerevisiae* contain a functional mitochondrial protein synthesising system. Mitochondrial RNA has been isolated from anaerobically grown cells (Forrester, Watson and Linnane, 1971).

Exposure of anaerobic yeast cells to oxygen results in a lag-free synthesis of components of the electron transfer chain and has also been reported to induce a burst of synthesis of mitochondrial DNA (Rabinowitz *et al.*, 1969). This has been reported to be due to a 20-fold increase in mitochondrial DNA polymerase activity (Rabinowitz *et al.*, 1969). Implicity, this observation suggests that anaerobiosis represses or inactivates mitochondrial DNA polymerase activity. Aeration also results in the appearance of new transcripts of mitochondrial DNA (Fukuhara, 1967).

It thus appears that as far as the mitochondrion is concerned anaerobiosis specifically inhibits the synthesis of the electron transfer chain and, upon aeration, or during respiratory adaptation synthesis of the electron transfer chain is specifically induced.

1-2-2 CATABOLITE REPRESSION OF ENZYME SYNTHESIS

Activities of respiratory enzymes are high when *S. cerevisiae* is cultured aerobically under conditions in which energy can only be derived from oxidative processes, e.g. in a culture medium containing lactate or ethanol as the sole carbon source. Correspondingly the cytology of this cell is characterized by the presence of numerous well-defined mitochondria with double membranes and cristae (Linnane, 1965). However, in the presence of high concentrations of readily fermented substrate such as glucose, the synthesis of mitochondrial enzymes (such as

ATPase, TCA cycle enzymes) is markedly inhibited (Strittmatter, 1957; Polakis and Bartley, 1965; Linnane, 1965; Tzagoloff, 1969) and a predominantly fermentative mode of metabolism prevails. The decrease in mitochondrial oxidative activity brought about by high concentrations of glucose in the growth medium is reflected in the cytology of the cell as shown by a decrease in number and definition of mitochondrial membranes and cristae (Linnane, 1965). In cultures of *S. cerevisiae* with glucose as the carbon source, synthesis of aerobic cytochromes is repressed as long as elevated levels of glucose remain in the medium. Cellular respiratory activity increases due to the *de novo* synthesis of respiratory enzymes upon exhaustion of the sugar. This developmental process, known as glucose derepression, has been utilized extensively for studies of organelle development (Roodyn and Wilkie, 1968; Henson *et al.*, 1968; Mahler *et al.*, 1968).

With galactose as carbon source, respiratory activity intermediate between glucose-repressed and derepressed is obtained, and this is accompanied by an increased number of mitochondria per cell. These are better defined compared with those seen in glucose-repressed cells (Polakis and Bartley, 1965; Linnane, 1965). The nature of the carbon source in the growth medium thus exerts control over the synthesis of respiratory enzymes in aerobically grown cells. In addition, the rate of respiratory adaptation of anaerobically grown cells is governed by catabolite repression. The presence of glucose in the adaptation medium retards the development of respiratory activity; this repressive effect is not as pronounced when galactose is used in place of glucose (Strittmatter, 1957; Tustanoff and Bartley, 1964; Linnane, 1965). Furthermore the presence of glucose during the anaerobic phase of growth has a severe retarding effect on subsequent respiratory development in the presence of oxygen, compared with galactose (Linnane, 1965).

The amount of DNA per mitochondrion in yeast was originally thought to vary with the extent of derepression of the culture. Thus mitochondrial DNA content in the cell of logarithmic phase culture was low or hardly

detectable and it increased correspondingly with the increase in respiratory activity of the cell (Moustacchi and Williamson, 1966). However this finding has recently been re-examined, and the difference is now ascribed to differential extractability of mitochondrial DNA from cells at different stages of growth (Williamson, 1970). Glucose-repressed and derepressed cells of *S. cerevisiae* contain approximately equal amounts of mitochondrial DNA.

In vertebrates the amount of RNA per mitochondrion is of the order of 10-20 times that of DNA but in unicellular organisms the ratio may vary with physiological conditions of the culture, such as glucose repression. The increase in RNA content of mitochondria can also be correlated with the increased activity of DNA-dependent RNA polymerase in mitochondria when yeast cells are released from glucose repression (South and Mahler, 1968).

Mitochondrial protein synthesis also appears to be affected by catabolite repression. Thus glucose-repressed cells have a less active mitochondrial protein synthesising system than glucose-derepressed cells as judged by *in vivo* amino acid incorporation studies (Kellerman, Griffith, Hansby and Linnane, 1971).

Though much is known about the effect of glucose repression, very little is known about its molecular mechanism. Recently 3'-5' cyclic AMP has been implicated in the regulation process of glucose repression (Van Wijl and Konijn, 1971). These workers found that intracellular cyclic AMP concentration parallels enzymic activities of succinic dehydrogenase. Thus the intracellular concentration of cyclic AMP is higher in derepressed cells of yeast than in glucose-repressed cells. Whether it is justified to implicate cyclic AMP in this regulatory process remains to be substantiated.

1-2-3 PETITE MUTATION AND MUTATIONS AFFECTING MITOCHONDRIAL FUNCTION

The above brief review has shown that mitochondria of *S. cerevisiae* have great variability in their enzyme constitution. They may be temporarily but not irreversibly

altered in response to environmental changes. However, mitochondrial function may also be permanently impaired as a result of mutation. A characteristic of *S. cerevisiae* is its ability to give rise to spontaneous mutants which are respiratory-deficient, so called "petite" mutants (Ephrussi, 1953). When wild-type and petite cells of opposite mating type are crossed, the mode of segregation of petite characteristic is non-Mendelian and follows the pattern of cytoplasmic inheritance. It has therefore been suggested that respiratory competence in *S. cerevisiae* is controlled by factors which reside in the cytoplasm (Ephrussi, 1953), and these have been equated with mitochondrial DNA (Reich and Luck, 1966). The spontaneous mutation frequency from wild-type yeast to petite mutants is high (about 10^{-3}) compared with chromosomal mutations (10^{-6} to 10^{-7}) but respiratory-deficient mutants can be produced at frequencies up to almost 100% by treatment with a number of chemical agents such as euflavine, ethidium bromide, tetrazolium salts and heavy metal ions such as Cu^{++}, Ni^{++}, Co^{++} and Mn^{++} (for review see Nagai *et al.*, 1961; Wilkie, 1966; Roodyn and Wilkie, 1968).

In addition to cytoplasmic mutants which are induced by the loss or inactivation of the cytoplasmic hereditary determinant, nuclear petites may be produced as a result of nuclear gene mutations (Ephrussi, 1953; Sherman, 1964). Spontaneous mutation and chemical treatment with agents described above generally give rise to cytoplasmic petites. UV irradiation, however, may produce cytoplasmic, nuclear, as well as double mutants in which mutations in both cytoplasmic determinant and nuclear genes have taken place (Ephrussi, 1953; Raut, 1954; Wilkie, 1966).

The euflavine-induced petite is characterized by the absence of cytochromes aa_3, b and c_1, and lower levels of TCA cycle enzymes. As a result the petite relies solely on fermentation for energy metabolism and hence has a slower rate of growth, compared with wild-type yeast, when fermentable substrate becomes limiting (Ephrussi, 1953). Because petites require fermentable substrate for growth, and because fermentable substrates generate a catabolite

repression (though of varying intensity depending on the substrate used), care needs to be exercised in the interpretation of results obtained from experiments carried out with petites. Effects due to glucose repression or petiteness are difficult to dissociate. Accompanying the enzymic changes are structural alterations of the mitochondria (Yotsuyanagi, 1962). The organelle contains reduced numbers of cristae and appears to be much larger in size but fewer in number compared with those of the wild-type yeast grown under glucose-derepressed conditions. As the cytoplasmic petite and the wild-type yeast are isogenic with respect to nuclear genes, it follows that the cytoplasmic determinant directly controls the synthesis of a number of the mitochondrial respiratory enzymes and presumably the formation of some of the structural components of membranes. It is relevant to note that cytoplasmic mutants retain the ability to synthesize cytochrome *c*. Mutants unable to synthesize cytochrome *c* are affected by segregational or nuclear mutations only, which thus implies that the synthesis of cytochrome *c* (apoprotein and heme prosthetic group) is under the control of nuclear genes. Indeed, Sherman (1964) found that six different loci controlled the cellular concentration of cytochrome *c*, with one locus being the structural gene for cytochrome *c* and other loci being regulatory. Apart from cytochrome *c*, the segregational and cytoplasmic mutants share many biochemical properties.

Since the original discovery of Ephrussi (1953) that mutation to respiratory deficiency in yeast is due to loss or inactivation of a cytoplasmic factor, numerous attempts have been carried out to test the possibility that such mutations involve loss or modification of the mitochondrial DNA. Corneo *et al*. (1966), Moustacchi and Williamson, (1966) and Tewari *et al*. (1966) reported that petite cells grown in a conventional shake culture were essentially devoid of mitochondrial DNA, although this DNA was readily detected in the corresponding wild-type strain grown under identical conditions. This suggested that the petite mutation involved the permanent loss of mitochondrial DNA.

However, the validity of these experimental observations was subsequently contested by Mounolou *et al.* (1966) who argued that glucose repression of mitochondrial DNA in respiration-deficient cells had not been excluded. In support of this view, Mounolou *et al.* found essentially normal amounts of a mitochondrial-type DNA in petite cells grown in a chemostat culture which minimized repression by glucose. However, petite mitochondrial DNA differed from that present in the wild-type cells in being of lower buoyant density in cesium chloride gradients. No density difference was detected between the nuclear DNA of the petite and wild-type, or between mitochondrial DNA species from the wild-type and a segregational respiration-deficient mutant. Mounolou *et al.* concluded, therefore, that the "petite" mutation resulted in the modification, rather than the loss, of mitochondrial DNA.

The findings of Mounolou *et al.* (1966) have been confirmed by Mehotra and Mahler (1968). However, the effect of glucose repression on mitochondrial DNA has now been clarified by Williamson (1970) and Fukuhara (1969). It is now said to be due primarily to effects on the extractability of mitochondrial DNA and not to a repression of replication or synthesis of mitochondrial DNA as Mounolou *et al.* (1966) envisaged.

The continued replication of mitochondrial DNA in the absence of mitochondrial protein synthesis (i.e. in chloramphenicol inhibited cells) (Davey, Yu and Linnane, 1969) and in petites (whose mitochondrial DNA is composed almost entirely of adenine and thymine nucleotides and hence has a very restricted information content) indicates that the polymerase cannot be coded for by mitochondrial DNA (Perlman and Mahler, 1970).

It has been claimed by Wintersberger (1967) that petite cells have no mitochondrial RNA (high molecular weight RNA), but retain low molecular weight RNA (presumably tRNA). However, Fukuhara *et al.* (1969) concluded from hybridization experiments that high molecular weight RNA is also transcribed from petite mitochondrial DNA. Furthermore, mitochondria from a cytoplasmic petite mutant

of *S. cerevisiae* have been reported to contain almost five times less RNA than mitochondria from the corresponding wild-type strain (Wintersberger, 1967). However, this could be due to instability of petite mitochondrial membranes, and hence loss of RNA during isolation. It might also be a consequence of glucose repression.

As mentioned above the non-respiring mitochondria of petite mutants appear to lack ribosomal RNA and normal mitochondrial DNA; apparently, they contain a defective protein synthesizing system permanently blocked at the transcription level. In many other biochemical properties, however, the mitochondria of the mutant resemble those of chloramphenicol-repressed wild-type cells. Thus, they have few, if any, distinct cristae, lack cytochromes aa_3 and *b*, but contain almost normal or elevated levels of cytochrome *c*, fumarase, malate dehydrogenase, and other easily extractable mitochondrial enzymes (Clark-Walker and Linnane, 1967). According to Kovac and Weissova (1968) and Schatz (1970), they also contain near-normal amounts of ATPase. However, the enzyme associated with the mutant mitochondria is cold-labile and insensitive to oligomycin, and differs in these respects from the enzyme bound to the wild-type mitochondria which is cold-stable and very sensitive to oligomycin.

Not all cytoplasmically-inherited mutations in yeast are respiratory deficient mutations. Cytoplasmically-inherited mutants with altered drug sensitivity but retaining respiratory competence have been obtained. Normally when wild-type yeast is cultured in the presence of erythromycin or chloramphenicol, synthesis of respiratory cytochromes is specifically inhibited. This suppression is due to inhibition by erythromycin or chloramphenicol of the mitochondrial protein synthesising system. Recently, Thomas and Wilkie (1968) and Linnane *et al.* (1968b) have isolated several mutants which differ from normal parent strains in that synthesis of cytochromes is no longer inhibited by these drugs. Some of these mutants are permeability mutants and are due to chromosomal mutations, but some are cytoplasmically-inherited, and result in an increased

resistance of the mitochondrial protein synthesising system to inhibition by erythromycin.

Thus, it is apparent that because of the availability and nonlethal nature of mutations affecting different aspects of mitochondrial function in *S. cerevisiae*, genetic analysis of mitochondrial differentiation can be carried out.

1-2-4 RATIONALE FOR THE WORK UNDERTAKEN IN THIS THESIS

S. cerevisiae is unique in having all of the advantages of manipulation of mitochondrial genome and function in combination. Furthermore, *S. cerevisiae* has a short generation time and therefore a large quantity of cell material can be obtained. In contrast, mammalian cells either *in situ* or in tissue cultures have relatively long generation times. Unlike *S. cerevisiae*, variation of mitochondrial differentiation in mammalian cells is difficult to control because of homeostatic mechanisms, and the absence of definite extrinsic factors such as catabolite repression and anaerobiosis for manipulation. Complication also arises from the fact that mammalian cells cannot tolerate gross modification to their mitochondrial function upon which they are obligatorily dependent. Even for eucaryotes on the lower end of the evolutionary scale, organisms with definite environmental and genetic controls of mitochondrial function are rare. Obligate aerobes are generally petite-negative, i.e. incapable of generating stable respiratory deficient mutants, characterized by the absence of glucose repression and anaerobiosis (Bulder, 1964 a, b; De Deken, 1966; Kellerman *et al.*, 1969; Heslot *et al.*, 1970 a, b). For these reasons many studies concerning the regulation and mode of synthesis of respiratory enzymes of mitochondria are therefore confined to systems offered by *S. cerevisiae*. It is in a sense unfortunate because much of the knowledge concerning the synthesis and control of mitochondrial function has been obtained from an organism which does not require classical mitochondrial function for survival. Therefore it may be that conclusions obtained from studies

of *S. cerevisiae* ought not be extrapolated to, or may not have relevance to, the situation in obligate aerobes such as mammalian cells. The validity of this statement can be tested in a comparative study of a facultative anaerobe (such as *Saccharomyces cerevisiae*) and an obligate aerobe of approximately equivalent complexity of cellular structure and function.

It is obvious that for a comparative study to be meaningful it is necessary to compare organisms under identical conditions. Therefore, in finding a candidate for comparison with the facultative anaerobe *Saccharomyces cerevisiae* an important consideration is that the candidate should be amenable to culture and study in identical environments. *Candida parapsilosis* appeared to be suitable in these respects. It is an obligate aerobe, grows in aerobic environments at rates similar to those of *Saccharomyces cerevisiae* and has a cellular morphology almost indistinguishable from that of aerobically cultured *Saccharomyces cerevisiae*. Furthermore, techniques and problems associated with organelle fractionation and purification are quite comparable in both organisms. Experiments on these two organisms can therefore be carried out under identical conditions.

The work undertaken and described in this thesis therefore represents an attempt to gain a deeper understanding of the mode and regulation of mitochondrial differentiation in the obligate aerobe *Candida parapsilosis*. By comparing experimental observations from *Candida parapsilosis* with well-documented observations from *Saccharomyces cerevisiae*, conclusions can be drawn or should become obvious as to the generality and applicability of results obtained from studies of *Saccharomyces cerevisiae* to mitochondrial differentiation in other organisms. The work undertaken includes examination of the respiratory physiology and mitochondrial genetic apparatus of these two organisms as well as the effect of inhibitors of protein and nucleic acid synthesis on aspects of mitochondrial differentiation.

1-2-5 A NOTE ON TERMINOLOGY

It has been customary to call mitochondrial precursors

in anaerobic cells of yeast 'promitochondria' by analogy with proplastids, precursors of chloroplasts, found in dark grown plant tissues (Schatz and Saltzgaber, 1969). As discussed before when 'promitochondria' are examined for known catalytic functions and structural features of mitochondria of aerobic cells, it is found that except for the absence of the electron transfer chain (classical mitochondrial function), promitochondria are equipped with most if not all catalytic and structural features of aerobic mitochondria, though there may be secondary effects on amounts of other enzymes present (Schatz, 1970). Because of the importance and diversity of its metabolic functions the mitochondrion is incompletely defined by its activity of oxidative phosphorylation. The term "promitochondrion" is therefore inappropriate. It seems more appropriate to consider units of specialized cytoplasm, organized within mitochondrial membranes around the mitochondrial DNA of anaerobic cells, as mitochondria lacking some components found in mitochondria of aerobic cells. Although terminologically convenient the term "promitochondrion" must be considered redundant.

Studies of changes that occur in mitochondria after the transfer of yeast cells from an anaerobic to an aerobic environment, or from high glucose repression to low glucose repression are frequently designated as "biogenesis of mitochondria". Under these conditions, only the electron transfer chain (and possibly certain phosphorylation proteins) are preferentially synthesized. The term "biogenesis" has also been used for studies of the synthesis of a few mitochondrial components in animal cells. However, since what is being studied in each case is a single component or a few components of the whole organelle, these studies do not concern the synthesis of new mitochondria. Changes that occur when yeast cells are derepressed or exposed to oxygen are therefore more appropriately described as an elaboration of pre-existing structure and function, hence "mitochondrial differentiation". In fact Plattner *et al.* (1970) have demonstrated the physical continuity of mitochondria in the anaerobic-aerobic transition of *Saccharomyces*.

Mitochondrial components may be formed *de novo*, but formation of new organelles is beyond doubt a result of growth and division of pre-existing mitochondria. However, even this mode of multiplication of mitochondria may not be a complete description, since mitochondria have been seen to fragment and coalesce (Frederic, 1958). Hence, it is conceivable that there exists a "pool of mitochondrial elements" divided at any given time into many single organellar units, but coalescing and sub-dividing, being added to and degraded, in such a way as to make the concept of an "individual" mitochondrion quite inappropriate.

CHAPTER 2

A COMPARATIVE STUDY OF THE RESPIRATORY PHYSIOLOGY OF *C. PARAPSILOSIS* AND *S. CEREVISIAE* (1) EFFECT OF CAP AND ER.

CHAPTER 2

2-1 INTRODUCTION

Selective inhibition of the synthesis of cytochromes aa_3, b, and c_1 of the electron transfer chain in *Saccharomyces cerevisiae* can be effected by growing cells in the presence of specific inhibitors of the mitochondrial protein-synthesising system (MPSS) i.e. chloramphenicol (CAP) or erythromycin (ER). These cells however still contain other carriers of the electron transfer chain such as coenzyme Q and cytochrome *c* (Linnane *et al.*, 1968; Gordon and Stewart, 1971). Respiratory activity of such cells is low.

In this chapter, I propose to describe aspects of the respiratory physiology and effects of CAP and ER on the synthesis of cytochromes of the classical electron transfer chain in *Candida parapsilosis*. A comparison of results obtained with *Saccharomyces cerevisiae* and *Candida parapsilosis* will be made and discussed.

2-2 EXPERIMENTAL

2-2-1 ORGANISMS

The obligate aerobe used throughout experiments described in this thesis was identified as *Candida parapsilosis* (Ashf) *Lange* et *Guerra* and the facultative anaerobe *Saccharomyces cerevisiae* was a locally-isolated diploid called strain M (Linnane *et al.*, 1968). Both organisms were maintained on agar slopes containing yeast extract (1% w/v), peptone (2% w/v) and glycerol (10% v/v). Before inoculation into the culture flask both were propagated on liquid medium of the same composition as that used in growth flasks.

2-2-2 CULTURE MEDIUM

(i) *Saccharomyces* medium

The composition of the medium used for cultures of *C. parapsilosis* and *S. cerevisiae* was constituted as follows:

	(% w/v)
Difco yeast extract	0.5
$(NH_4)_2SO_4$	0.12
NaCl	0.05
$CaCl_2$	0.01
$MgSO_4.7H_2O$	0.2
KH_2PO_4	0.1
$FeCl_3$	0.0005
Glucose or glycerol	2.5

Further additions or omissions to this basic medium will be specified in the text.

(ii) *Candida utilis* medium

In experiments investigating factors inducing the development of an alternative pathway of oxidation in *C. parapsilosis*, another medium described for growth of *C. utilis* was used. This had the following composition (Osmond and Rees, 1969).

	Weight per litre
$MgSO_4.7H_2O$	1.5 gm
KH_2PO_4	1.0 gm
NaCl	0.5 gm
$ZnSO_4.7H_2O$	0.1 gm
$FeCl_3.6H_2O$	50 mg
$CuSO_4.5H_2O$	10 mg
$Na_2B_4O_7.10H_2O$	0.5 mg
$MnSO_4.4H_2O$	0.1 mg
$Na_2MoO_4.2H_2O$	0.1 mg
Inositol	0.8 mg
Pyridoxine hydrochloride	1.5 mg
Thiamine hydrochloride	4.0 mg
Calcium pantothenate	0.2 mg
Biotin	0.05 mg
Difco yeast extract	10 gm
Glucose or glycerol	25 gm or 50 gm

2-2-3 CULTURE CONDITIONS

Cultures were inoculated with a standard initial inoculum of 0.03 mg dry weight of cells per ml of liquid culture. Cultures were grown at 29°C in conical flasks

on a rotary shaker table (New Brunswick) at approximately 200 rpm. Culture volumes were limited to one-quarter to one-third of the capacity of the containing vessel.

2-2-4 DETERMINATION OF GROWTH OF CELLS

Growth of cells in culture was determined by light scattering at 640mμ of a suitably diluted suspension of cells. Within appropriate limits, light scattering at this wavelength corresponds proportionally to dry weight of cells as determined by a gravimetric method.

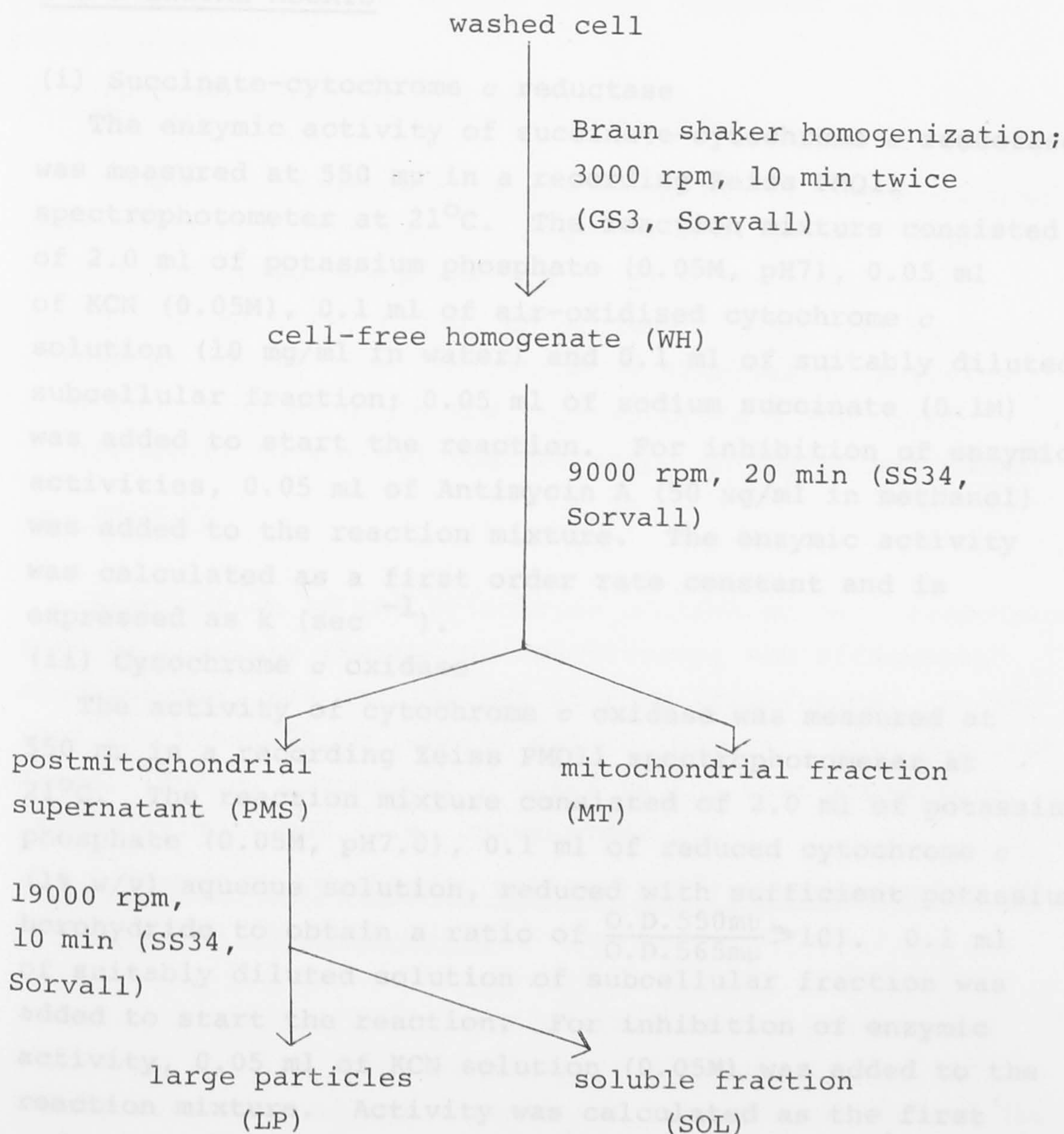
2-2-5 ABSOLUTE CYTOCHROME ABSORPTION SPECTRA OF WHOLE CELLS

Absorption bands of hematin compounds in cells of both *C. parapsilosis* and *S. cerevisiae* were examined with a Cary 14 spectrophotometer fitted with a scattered transmission accessory. A thick suspension of cells (standard concentration of 14 mg dry weight of cells per ml) in 50% glycerol (v/v) was placed in the sample cuvette, and treated with a few grains of sodium dithionite to ensure reduction of hematin compounds. All samples were compared with a reference cuvette containing a suspension of flour particles in starch gel, adjusted to have an opacity similar to that of the cell suspension (Linnane *et al.*, 1968).

2-2-6 CELL FRACTIONATION

For enzymic assay and difference spectroscopy of subcellular fractions, cells were disrupted and fractionated as follows. After harvesting by centrifugation, cells were washed twice with distilled water, once with STE (0.5M sorbitol, 0.01M Tris HCl pH 7.4, sodium EDTA 0.5mM pH7.4), and resuspended in one volume of STE per volume of packed cells. The cell suspension was mixed with one volume of glass beads (diameter 0.45-0.5mm) per volume of cell suspension, and broken in a Braun MSK homogenizer operated at maximum speed for 25 seconds, with carbon dioxide as coolant (Schatz, 1967). The brei was decanted and

centrifuged twice at 3000 rpm for 10 min (Sorvall, GS3) to remove cell debris. The cell-free homogenate from the second centrifugation was then centrifuged at 9000 rpm for 20 min (Sorvall, SS34) to sediment a mitochondrial fraction (MT). The supernatant was denoted postmitochondrial supernatant (PMS). The PMS was centrifuged at 19000 rpm for 10 min (Sorvall, SS34) to sediment large membrane fragments. The resultant pellet was called large particles (LP) and the supernatant, soluble (SOL) fraction. The procedure may be summarized thus:



2-2-7 DIFFERENCE SPECTRA

Specific cytochrome content of cell-free homogenates and subcellular fractions was determined from the oxidized (potassium ferricyanide) versus reduced (sodium dithionite) spectrum using the extinction coefficients of Estabrook and Holowinsky (1961).

2-2-8 PROTEIN DETERMINATION

Protein contents of cell-free homogenates and subcellular fractions were determined by the method of Lowry, Rosebrough, Farr and Randall (1951).

2-2-9 ENZYME ASSAYS

(i) Succinate-cytochrome *c* reductase

The enzymic activity of succinate-cytochrome *c* reductase was measured at 550 mμ in a recording Zeiss PMQ11 spectrophotometer at 21°C. The reaction mixture consisted of 2.0 ml of potassium phosphate (0.05M, pH7), 0.05 ml of KCN (0.05M), 0.1 ml of air-oxidised cytochrome *c* solution (10 mg/ml in water) and 0.1 ml of suitably diluted subcellular fraction; 0.05 ml of sodium succinate (0.1M) was added to start the reaction. For inhibition of enzymic activities, 0.05 ml of Antimycin A (50 μg/ml in methanol) was added to the reaction mixture. The enzymic activity was calculated as a first order rate constant and is expressed as k (sec $^{-1}$).

(ii) Cytochrome *c* oxidase

The activity of cytochrome *c* oxidase was measured at 550 mμ in a recording Zeiss PMQ11 spectrophotometer at 21°C. The reaction mixture consisted of 2.0 ml of potassium phosphate (0.05M, pH7.0), 0.1 ml of reduced cytochrome *c* (1% w/v) aqueous solution, reduced with sufficient potassium borohydride to obtain a ratio of $\frac{\text{O.D.550m}\mu}{\text{O.D.565m}\mu} \geqslant 10$). 0.1 ml of suitably diluted solution of subcellular fraction was added to start the reaction. For inhibition of enzymic activity, 0.05 ml of KCN solution (0.05M) was added to the reaction mixture. Activity was calculated as the first order rate constant, and is expressed as k (sec $^{-1}$).

2-2-10 ELECTRON MICROSCOPY OF CELLS

For electron microscopy, washed cells were fixed in potassium permanganate, stained with uranyl nitrate and potassium dichromate, embedded in prepolymerized methacrylate, sectioned, and then examined in a Hitachi HU 11E electron microscope, as described by Wallace and Linnane (1964).

2-2-11 GLUCOSE ASSAY

Concentrations of glucose in culture media were estimated using Glucostat reagent (Worthington Biochemical Corporation), and the second method described by the manufacturer.

2-2-12 OXYGEN UTILIZATION BY WHOLE CELLS

Oxygen utilization by whole cells was measured polarographically at 30°C using a Clark-type electrode. The reaction mixture contained 2.0 ml of 0.05M potassium phosphate (pH 7.0), 0.1 ml of glucose (0.1M), 2-5 mg dry weight of cells. For inhibition of respiratory activity, 0.05 to 0.1 ml of 0.05M KCN, or Antimycin A (50 μg/ml in methanol) to a final concentration of 1μg/ml was added.

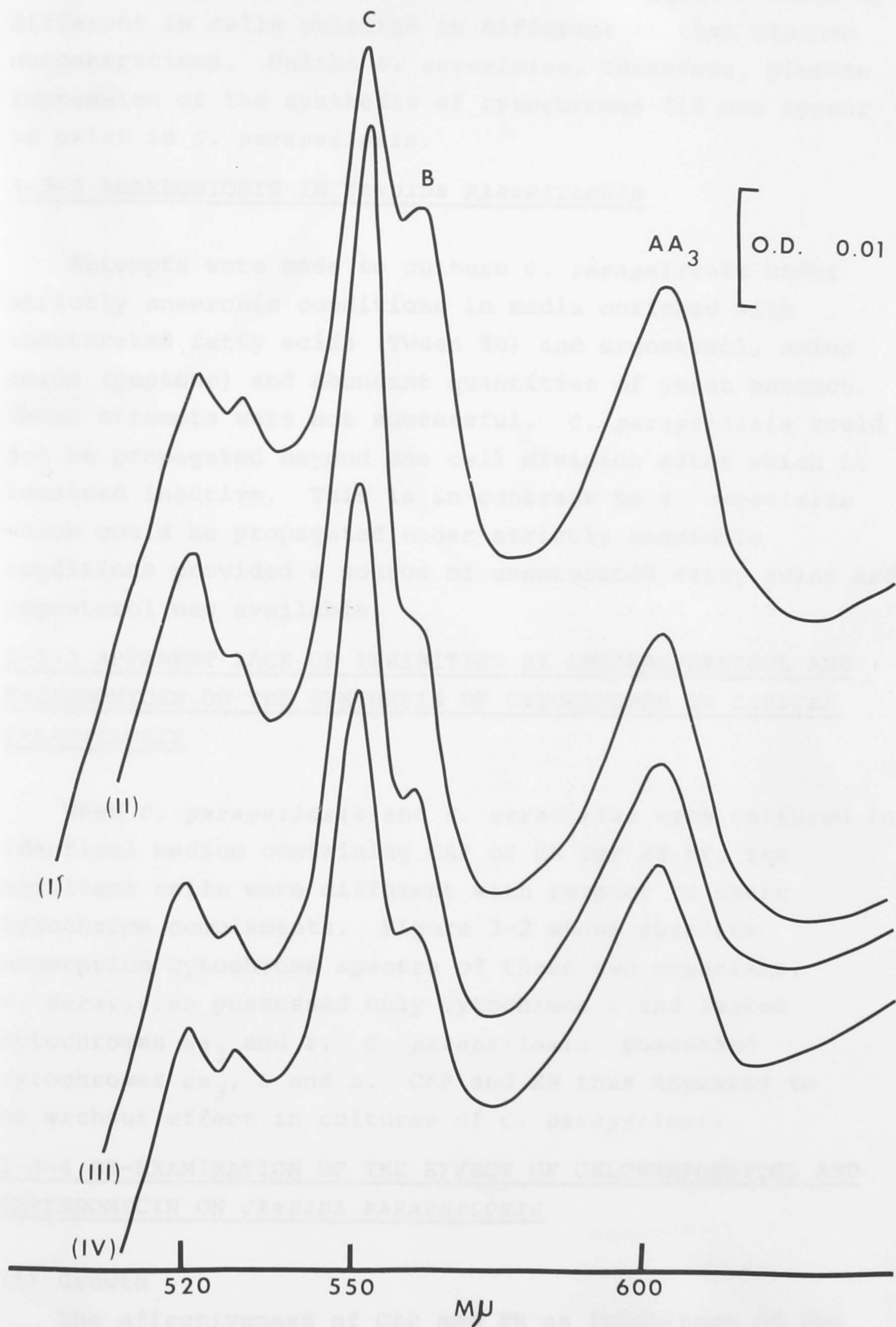
2-3 RESULTS

Examination of the cytochrome content of cell suspensions, both by absolute absorption spectroscopy and difference spectroscopy, was carried out at room temperature. It was thus not possible to resolve cytochromes $c+c_1$ into distinct bands. Hence, cytochrome *c* cited in the text represents *c*-type cytochromes (i.e. cytochromes $c+c_1$).

2-3-1 EFFECT OF GLUCOSE ON THE SYNTHESIS OF CYTOCHROMES OF THE ELECTRON TRANSFER CHAIN

When *C. parapsilosis* was cultured in the presence of different initial concentrations of glucose, little effect on the synthesis of cytochromes was seen (Figure 2-1). The

Figure 2-1. Absolute cytochrome absorption spectra of cells of *C. parapsilosis* cultured for 24 hr in *Saccharomyces* media containing initially 1% (curve I), 5% (curve II), 10% (curve III) and 20% (curve IV) glucose (w/v).
Peaks denoted by AA_3, B and C represent α-absorption bands of cytochromes aa_3, b and c respectively. Examination of absorption spectra was carried out at room temperature, hence peak C represents unresolved cytochromes $c+c_1$. Cell suspensions of all samples were reduced with sodium dithionite and examined at a standard concentration of 14 mg dry wt/ml.


C
B
AA3
O.D. 0.01
(I)
(II)
(III)
(IV)
520
550
600
MU

relative proportion of these cytochromes might be slightly different in cells cultured in different initial glucose concentrations. Unlike *S. cerevisiae*, therefore, glucose repression of the synthesis of cytochromes did not appear to exist in *C. parapsilosis*.

2-3-2 ANAEROBIOSIS IN *CANDIDA PARAPSILOSIS*

Attempts were made to culture *C. parapsilosis* under strictly anaerobic conditions in media enriched with unsaturated fatty acids (Tween 80) and ergosterol, amino acids (peptone) and abundant quantities of yeast extract. These attempts were not successful. *C. parapsilosis* could not be propagated beyond one cell division after which it remained inactive. This is in contrast to *S. cerevisiae* which could be propagated under strictly anaerobic conditions provided a source of unsaturated fatty acids and ergosterol was available.

2-3-3 APPARENT LACK OF INHIBITION BY CHLORAMPHENICOL AND ERYTHROMYCIN ON THE SYNTHESIS OF CYTOCHROMES IN *CANDIDA PARAPSILOSIS*

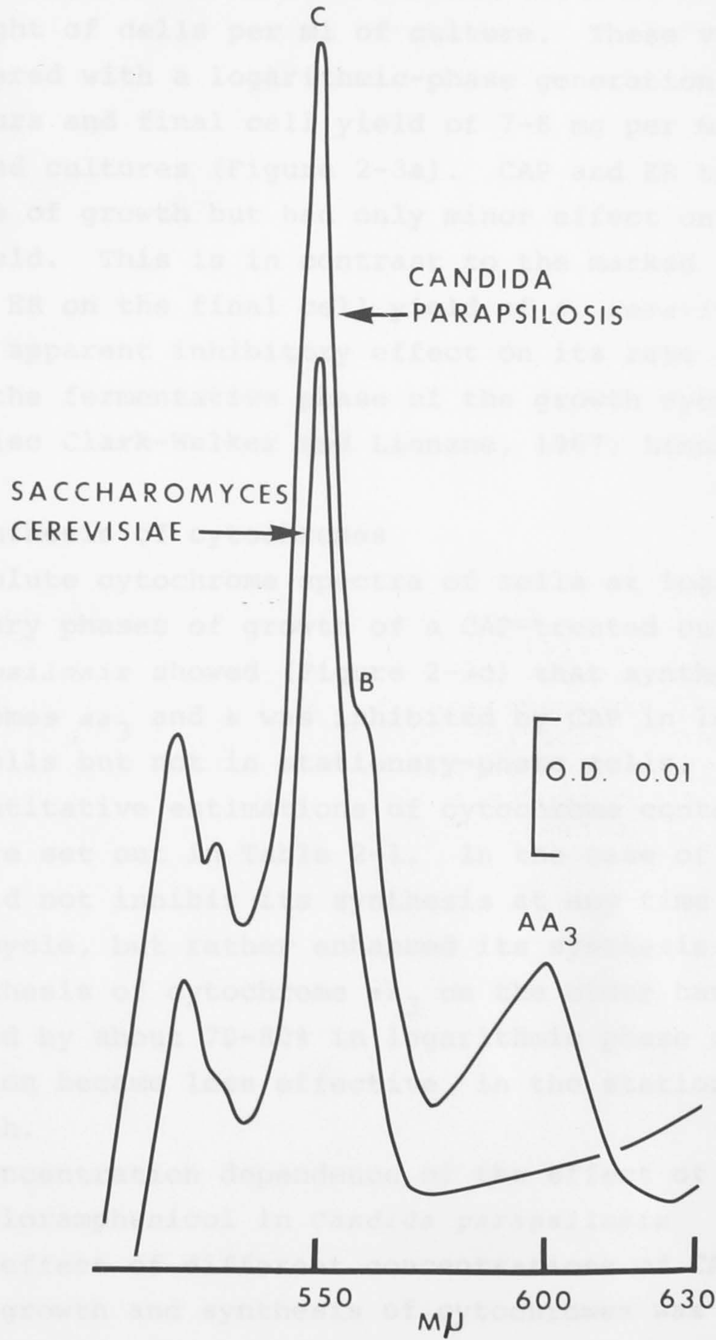
When *C. parapsilosis* and *S. cerevisiae* were cultured in identical medium containing CAP or ER for 24 hr, the resultant cells were different with respect to their cytochrome complements. Figure 2-2 shows absolute absorption cytochrome spectra of these two organisms. *S. cerevisiae* possessed only cytochrome *c* and lacked cytochromes aa_3 and *b*. *C. parapsilosis* possessed cytochromes aa_3, *b* and *c*. CAP and ER thus appeared to be without effect in cultures of *C. parapsilosis*.

2-3-4 RE-EXAMINATION OF THE EFFECT OF CHLORAMPHENICOL AND ERYTHROMYCIN ON *CANDIDA PARAPSILOSIS*

(i) Growth

The effectiveness of CAP and ER as inhibitors of the synthesis of cytochromes aa_3, *b* and *c* were examined more closely throughout the growth cycle of a CAP- or ER-treated culture of *C. parapsilosis*, in particular at

Figure 2-2. Absolute cytochrome absorption spectra of cells of *C. parapsilosis* and *S. cerevisiae* cultured for 24 hr in *Saccharomyces* medium containing 2.5% glucose (w/v) and 4 mg/ml of chloramphenicol. Peaks denoted by AA_3, B and C represent α-absorption bands of cytochromes aa_3, *b* and *c* respectively. Samples were examined at room temperature after reduction with sodium dithionite and at a standard cell density of 14 mg/ml.


C
CANDIDA
PARAPSILOSIS
SACCHAROMYCES
CEREVISIAE
B
O.D. 0.01
AA3
550
600
630
mμ

logarithmic and stationary phases (Figure 2-3). In the presence of CAP (4mg/ml) or ER (5-10 mg/ml), cells grew with a logarithmic-phase generation-time of 1.6 hr and a final cell yield (which was reached after 24 hr) of 6-7 mg dry weight of cells per ml of culture. These values may be compared with a logarithmic-phase generation-time of 1.25 hours and final cell yield of 7-8 mg per ml in untreated cultures (Figure 2-3a). CAP and ER thus affected the rate of growth but had only minor effect on the final cell yield. This is in contrast to the marked effect of CAP and ER on the final cell yield of *S. cerevisiae* but lack of apparent inhibitory effect on its rate of growth during the fermentative phase of the growth cycle (Figure 2-3b; also Clark-Walker and Linnane, 1967; Linnane *et al.*, 1968).

(ii) Synthesis of cytochromes

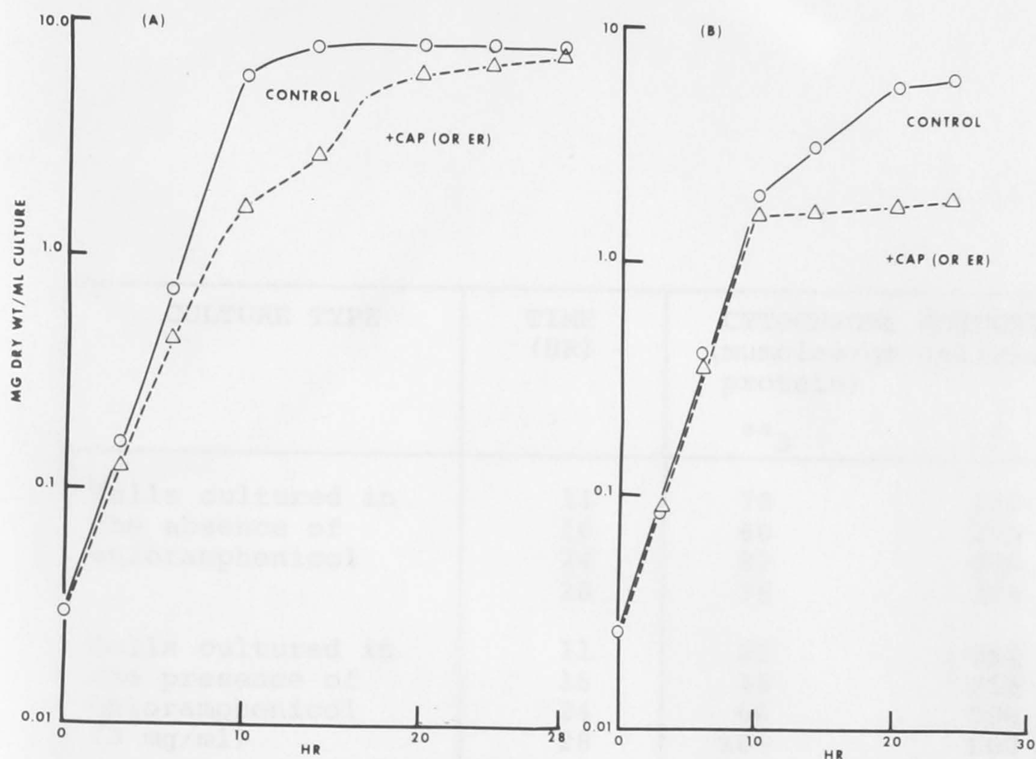
Absolute cytochrome spectra of cells at logarithmic and stationary phases of growth of a CAP-treated culture of *C. parapsilosis* showed (Figure 2-3c) that synthesis of cytochromes aa_3 and b was inhibited by CAP in logarithmic-phase cells but not in stationary-phase cells.

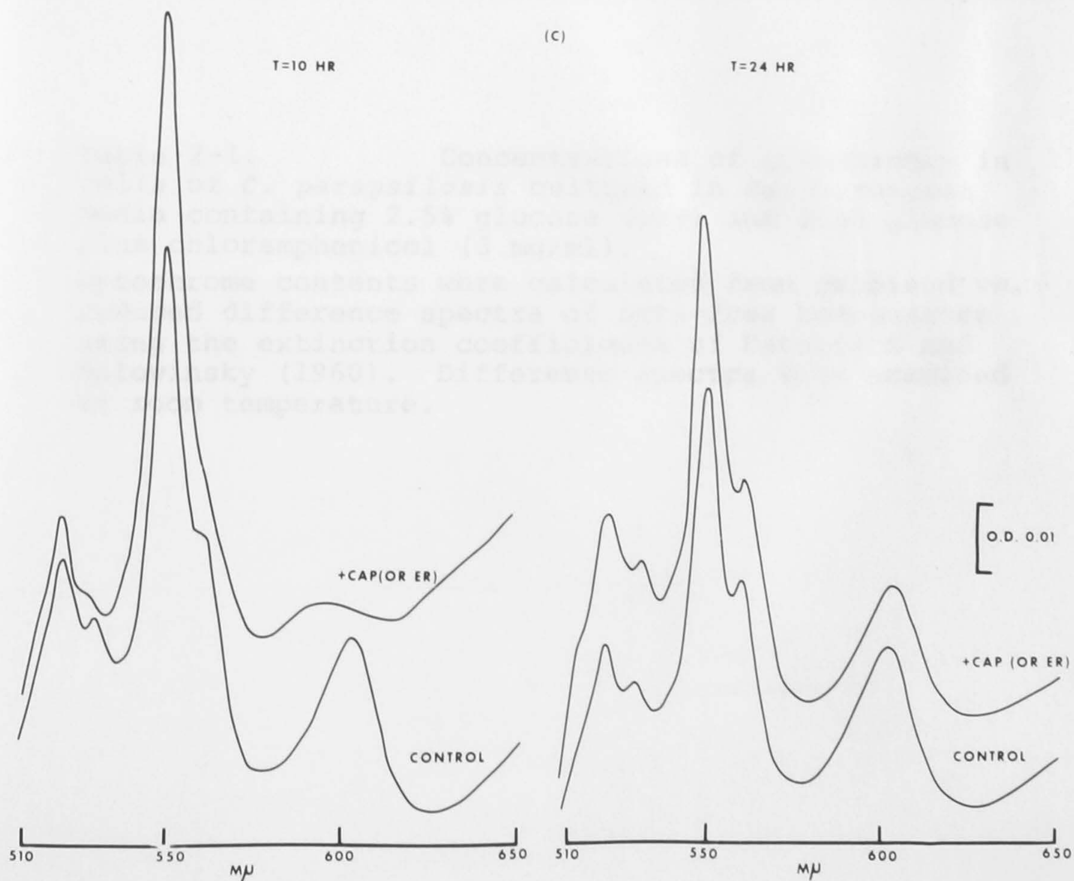
Quantitative estimations of cytochrome content of these cells are set out in Table 2-1. In the case of cytochrome c CAP did not inhibit its synthesis at any time of the growth cycle, but rather enhanced its synthesis about 2-fold. The synthesis of cytochrome aa_3 on the other hand was inhibited by about 70-80% in logarithmic phase cells; the inhibition became less effective in the stationary phase of growth.

(iii) Concentration dependence of the effect of chloramphenicol in *Candida parapsilosis*

The effect of different concentrations of CAP and ER on cell growth and synthesis of cytochromes was examined, and results are shown in Figure 2-4. With CAP at 2 mg/ml and above (for ER, 5 mg/ml and above), the rate of cell growth and the logarithmic-phase synthesis of cytochromes aa_3 and b were affected, whereas at CAP concentrations below 2 mg/ml inhibition was found to be marginal.

Figure 2-3. Growth of *C. parapsilosis* (Figure 2-3A) and *S. cerevisiae* (Figure 2-3B) in *Saccharomyces* media containing 2.5% glucose (w/v) and 2.5% glucose (w/v) plus chloramphenicol (4 mg/ml) or erythromycin (5-10 mg/ml).
Growth of cultures were measured by light scattering at 640 mμ. Growth curves of the control and antibiotic-treated cultures are denoted by ○———○ and △------△ respectively.
Figure 2-3(C). Absolute cytochrome absorption spectra of cells of *C. parapsilosis* removed from the control and CAP-treated cultures at times T=10 and T=24 hr.
Cells at times T=10 and T=24 hr were in logarithmic and stationary phase of growth respectively.


(A)
MG DRY WT/ML CULTURE
10.0
1.0
0.1
0.01
CONTROL
+CAP (OR ER)
0
10
20
28
HR
(B)
10
1.0
0.1
0.01
CONTROL
+CAP (OR ER)
0
10
20
30
HR


(C)
T=10 HR
T=24 HR
O.D. 0.01
+CAP(OR ER)
CONTROL
+CAP (OR ER)
CONTROL
510
550
600
650
MU
510
550
600
650
MU

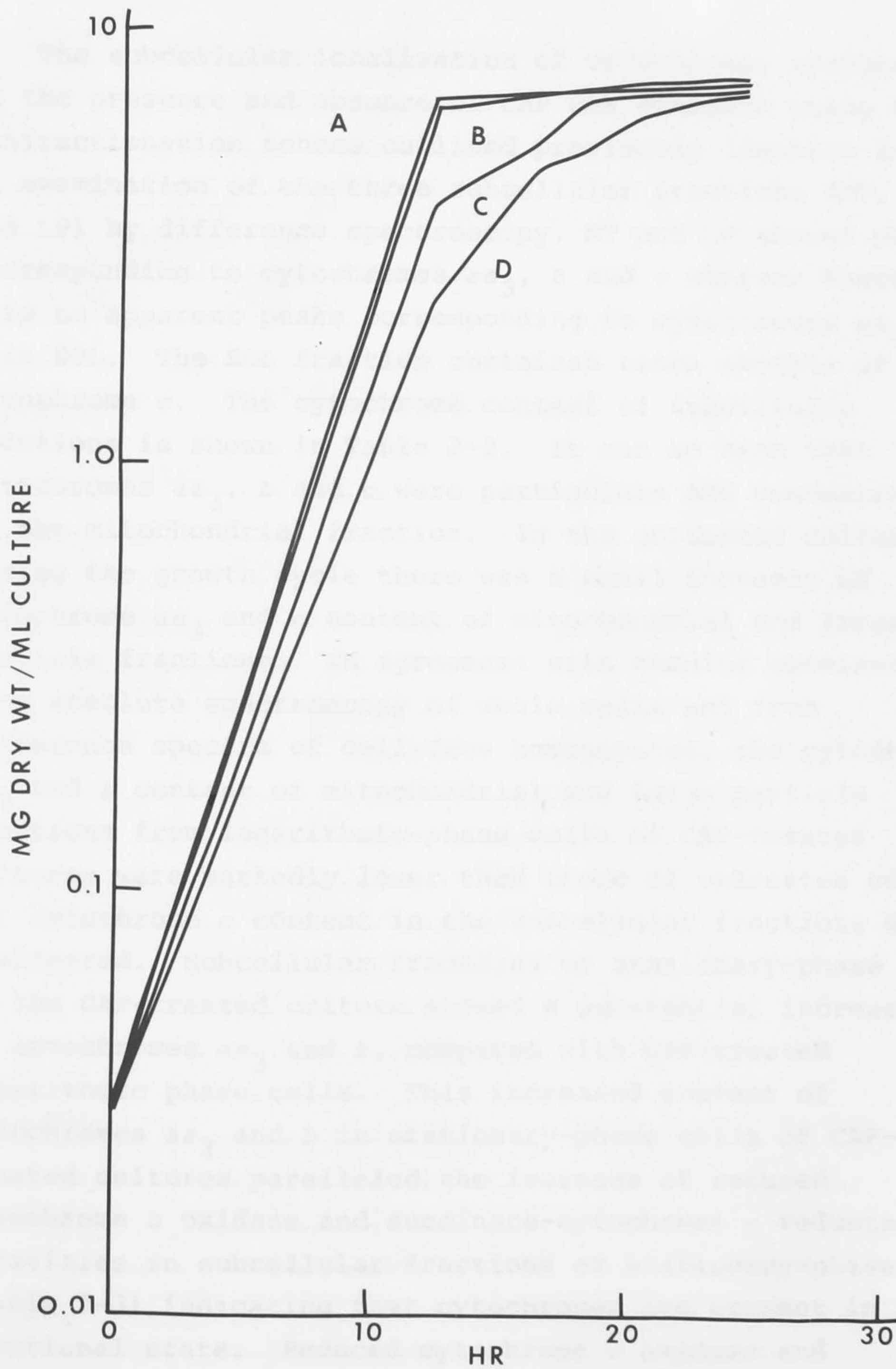
CULTURE TYPE	TIME (HR)	CYTOCHROME CONTENT (mμmoles/gm cellular protein)	
		aa_3	*c*
Cells cultured in the absence of chloramphenicol	11	78	147
	16	60	275
	24	82	270
	28	96	278
Cells cultured in the presence of chloramphenicol (3 mg/ml)	11	25	382
	16	48	518
	24	66	530
	28	106	660

Table 2-1. Concentrations of cytochromes in cells of *C. parapsilosis* cultured in *Saccharomyces* media containing 2.5% glucose (w/v) and 2.5% glucose plus chloramphenicol (3 mg/ml).

Cytochrome contents were calculated from oxidized vs. reduced difference spectra of cell-free homogenates using the extinction coefficients of Estabrook and Holowinsky (1960). Difference spectra were examined at room temperature.

Figure 2-4. Concentration dependence of inhibitory effect of CAP and ER.
Growth of *C. parapsilosis* was examined in *Saccharomyces* media containing 2.5% glucose (w/v) and chloramphenicol at 0.1, 0.2, 0.5, 1, 2, 3 and 4 mg/ml or erythromycin at 5 and 10 mg/ml. Insert shows a summary of examination of absolute cytochrome absorption spectra of cells of these cultures. + and - represent the presence and absence of the cytochrome respectively. The assignment of + and - was made relative to results obtained in Figure 2-3 (C). Thus, CAP-treated cells at time T=10 and T=24 hr of Figure 2-3 (C) were classified as aa_3- b- c+, and aa_3+ b+ c+ respectively.

GROWTH CURVE	SUMMARY OF EXAMINATION OF ABSOLUTE CYTOCHROME ABSORPTION SPECTRA OF CELLS						
	ADDITION TO CULTURE	CYTOCHROME PRESENT AT					
		T=12 HR			T=24 HR		
		aa_3	b	c	aa_3	b	c
A	Nil	+	+	+	+	+	+
A	CAP (0.1, 0.2, 0.5 mg/ml)	+	+	+	+	+	+
B	CAP (1 mg/ml)	+	+	+	+	+	+
C	CAP (2 mg/ml)	-	-	+	+	+	+
D	CAP (3, 4 mg/ml)	-	-	+	+	+	+
D	ER (5, 10 mg/ml)	-	-	+	+	+	+



MG DRY WT/ML CULTURE
10
1.0
0.1
0.01
A
B
C
D
0
10
20
30
HR

2-3-5 FUNCTIONAL STATES OF CYTOCHROMES SYNTHESIZED IN THE PRESENCE OF CHLORAMPHENICOL AND ERYTHROMYCIN

The subcellular localization of cytochromes synthesised in the presence and absence of CAP was examined using the subfractionation scheme outlined previously (Section 2-2-6). On examination of the three subcellular fractions (MT, SOL and LP) by difference spectroscopy, MT and LP showed peaks corresponding to cytochromes aa_3, *b* and *c* whereas there were no apparent peaks corresponding to cytochromes aa_3 and *b* in SOL. The SOL fraction contained trace amounts of cytochrome *c*. The cytochrome content of subcellular fractions is shown in Table 2-2. It can be seen that cytochromes aa_3, *b* and *c* were particulate and concentrated in the mitochondrial fraction. In the untreated culture during the growth cycle there was a small increase of cytochrome aa_3 and *c* content of mitochondrial and large particle fractions. In agreement with results obtained from absolute spectroscopy of whole cells and from difference spectra of cell-free homogenates, the cytochrome aa_3 and *b* content of mitochondrial and large particle fractions from logarithmic-phase cells of CAP-treated cultures were markedly lower than those of untreated cells, but cytochrome *c* content in the subcellular fractions were unaffected. Subcellular fractions of stationary-phase cells of the CAP-treated culture showed a substantial increase of cytochromes aa_3 and *b*, compared with CAP-treated logarithmic phase cells. This increased content of cytochromes aa_3 and *b* in stationary-phase cells of CAP-treated cultures paralleled the increase of reduced cytochrome *c* oxidase and succinate-cytochrome *c* reductase activities in subcellular fractions of stationary-phase cells (Table 2-3) indicating that cytochromes are present in a functional state. Reduced cytochrome *c* oxidase and succinate-cytochrome *c* reductase activities in the subcellular fractions were found to be completely sensitive to potassium cyanide and Antimycin A respectively.

It is also evident from Table 2-3 that a considerable amount of cytochrome *c* oxidase was not recovered in particulate

SUBCELLULAR FRACTION		CYTOCHROME CONTENT (mμmoles cytochrome/ gm protein)			
		a	a_3	b	c
MITOCHONDRIA					
CONTROL	T=12 HR	450	180	460	688
		490	182	448	730
	T=24 HR	490	365	475	823
		512	374	450	862
CAP-TREATED	T=12 HR	119	<10	119	623
		<10	<10	112	595
	T=24 HR	275	225	385	840
		204	138	244	870
LARGE PARTICLES					
CONTROL	T=12 HR	137	27	232	300
		154	144	209	297
	T=24 HR	190	200	245	500
		200	217	226	371
CAP-TREATED	T=12 HR	53	<10	86	597
		55	65	133	524
	T=24 HR	195	145	345	635
		186	224	265	763

TABLE 2-2. Cytochrome contents of subcellular fractions. Subcellular fractions of the control and CAP-treated cells of *C. parapsilosis* at times T=12 and 24 hr were obtained from corresponding cell-free homogenates by differential centrifugation to obtain mitochondrial and large particle fractions respectively as described in Section 2-2-6. Cytochrome contents were calculated from difference spectra of subcellular fractions. Pairs of figures given are results of two experiments. The symbol < means "less than".

CELL TYPE		SPECIFIC ENZYMIC ACTIVITIES OF SUBCELLULAR FRACTIONS		
		MITOCHONDRIA	LARGE PARTICLES	SOLUBLE
		cytochrome c oxidase		
CONTROL	T=12 HR	210±43	122±2	19
	T=24 HR	300±100	200±8	42
CAP-TREATED	T=12 HR	87±11	50±0	11
	T=24 HR	226±73	145±2	24
		succinate-cytochrome c reductase		
CONTROL	T=12 HR	60±4	43	↑
	T=24 HR	68±5	15	NOT
CAP-TREATED	T=12 HR	36±7	17	DETECTABLE
	T=24 HR	67±16	45	↓

Table 2-3. Enzymic activities of subcellular fractions.
Subcellular fractions were obtained from control and CAP-treated (3 mg/ml) cells of *C. parapsilosis* at times T=12 and T=24 hr by homogenization of cells with glass beads and differential centrifugation of resultant cell-free homogenates. Mitochondria and large particles were sediments of differential centrifugation of cell-free homogenates at 9000 rpm for 20 min and 19000 rpm for 10 min (Sorvall, SS34) respectively; soluble was the supernatant of the 19000 rpm centrifugation. Activities of cytochrome c oxidase and succinate-cytochrome c reductase were measured by the oxidation and reduction of cytochrome c at 550 mμ, respectively. Enzymic activities were calculated as first order rate constants ($k=sec^{-1}$) and expressed as k/gm protein of subcellular fraction. Data are results of one typical experiment. Enzyme assays were carried out in duplicates and data are given as average ± deviation. Cytochrome c oxidase was completely sensitive to 1mM KCN; the succinate-cytochrome c reductase was sensitive to 1μg/ml Antimycin A.

fractions. This is presumably a consequence of the mechanical method of cell fractionation; there was little difference between cell types in this respect.

These experiments show that the effectiveness of inhibition by CAP or ER of the synthesis of cytochromes aa_3 and b was in some way dependent on the growth phase in *C. parapsilosis*, whereas in *S. cerevisiae* the inhibition is independent of the phase of growth. In the growth cycle of a *Candida* culture, inhibition by CAP was seen only in logarithmic-phase i.e. in actively dividing cells; in stationary-phase i.e. in non-dividing cells, the antibiotic was largely ineffective.

2-3-6 CYTOLOGY OF *CANDIDA PARAPSILOSIS* CULTURED IN THE PRESENCE OF CHLORAMPHENICOL

An alternative method by which to measure variations of mitochondrial development and function is electron microscopy. Parameters such as the number of mitochondria per cell section and the number of cristae per mitochondrion may provide an indication of altered patterns of mitochondrial differentiation. The result of an analysis of many electron micrographs of cell sections is shown in Table 2-4. It shows distributions of the number of mitochondria per cell section, the number of cristae per mitochondrion, and the size and shape of these organelles in cells from different types of culture. Although small differences exist between the normal and CAP-treated cells in both logarithmic and stationary phases of growth with respect to the number of mitochondria per cell section, and size and shape of these mitochondria, differences appear to have only marginal significance. However, in respect of two features, the analysis showed quite marked differences. Firstly there were more mitochondria per cell section in stationary-phase (both in the normal and CAP-treated cultures) than in logarithmic-phase. Secondly, in the logarithmic-phase of growth, mitochondria in cells grown in the presence of CAP had fewer cristae than did mitochondria in untreated cells; cells cultured with or without CAP to stationary-phase of growth contained

CULTURE TIME (HR)	PARAMETERS ANALYSED														
	Number of mitochondria (with cristae) per cell section				Number of cristae per mitochondrion				Size of mitochondria in μ (long axis)				Shape of mitochondria		
	≤2	≤4	≤6	>6	≤2	≤4	≤6	>6	≤0.2	≤0.5	≤0.75	>0.75	Round and oval	Oblong	Elongated
T=11 HR															
CONTROL	0	20	70	10	15	42	42	0	37	46	9	8	51	44	5
CAP-TREATED	0	32	47	21	63	30	3	3	54	32	7	7	33	56	11
T=24 HR															
CONTROL	0	0	10	90	18	46	29	7	56	26	13	4	64	29	7
CAP-TREATED	0	2	24	74	22	46	28	4	40	45	13	2	65	30	5

Table 2-4. A summary of numerical analysis of electron micrographs of cell sections. Cells were removed from control and CAP-treated (3 mg/ml) cultures of *C. parapsilosis*, at times T=11 and T=24 hr, and processed for electron microscopy. Electron micrographs of cell sections were analysed in terms of the parameters listed in the table above. Results are expressed as percentage of individual parameters. Number of cell sections analysed was 100-150 for each cell type. Symbols ≤ and > mean "equal or less than" and "more than" respectively.

mitochondria with much the same number of cristae. These general features are illustrated in electron micrographs of cell sections shown in Figures 2-5, 2-6, 2-7 and 2-8. It seems therefore that the formation of cristae in the logarithmic-phase of growth is inhibited by CAP but this inhibition is not apparent in stationary-phase cultures. This phenomenon is correlated with the effect on the synthesis of cytochromes aa_3 and *b* in the logarithmic-phase of growth.

2-3-7 RESPIRATORY ACTIVITIES IN *CANDIDA PARAPSILOSIS* - EFFECT OF GROWTH PHASE AND ANTIBIOTICS

Observations cited above demonstrate a difference between *S. cerevisiae* and *C. parapsilosis* in their response towards inhibition by CAP or ER with respect to the synthesis of cytochromes aa_3 and *b*. This section describes another apparent difference in the respiratory physiology of these two organisms.

In normal cultures of *Saccharomyces* growing on a fermentable substrate such as glucose, the respiratory activity was low initially and progressively increased as the fermentable substrate became exhausted. The respiratory activity of *Saccharomyces* was almost completely cyanide and Antimycin A sensitive at all time of the growth cycle. In the presence of CAP, cellular respiratory activity was low and never developed significantly beyond levels obtained with anaerobically-grown cells. These are classical characteristics of this organism (Roodyn and Wilkie, 1968).

Respiratory activities of *C. parapsilosis* during a growth cycle in the presence or absence of CAP have been examined and results are shown in Figure 2-9. In sharp contrast to the increasing development of respiratory activity in *S. cerevisiae* with time, the respiratory activity of a normal culture of *C. parapsilosis* decreased abruptly in the logarithmic-phase of growth. Also, in sharp contrast to *S. cerevisiae* CAP did not significantly affect the cellular respiratory activity of *C. parapsilosis* at any time during the growth cycle.

Figure 2-5. Electron micrographs of sections of cells of a normal culture of *C. parapsilosis* at time T=11 hr.
Mitochondria are double membrane structures with infolding membranes. Magnification of these cell sections is 45000.

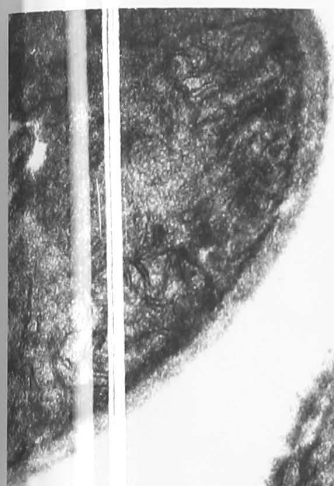
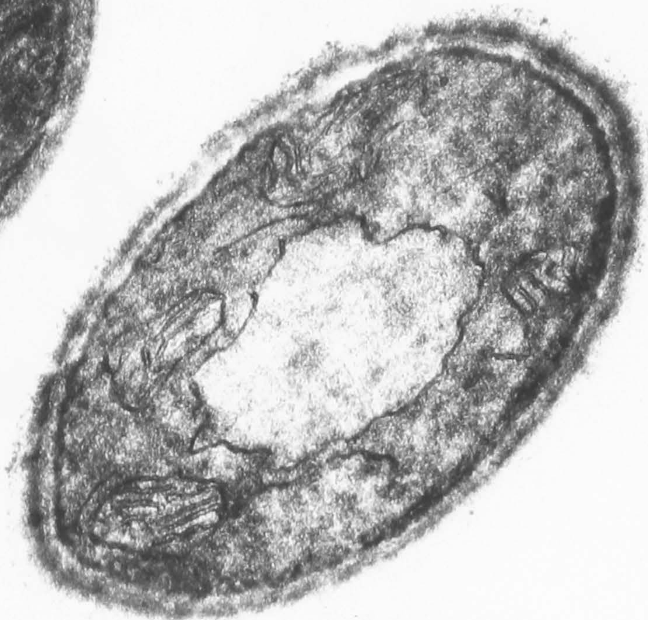
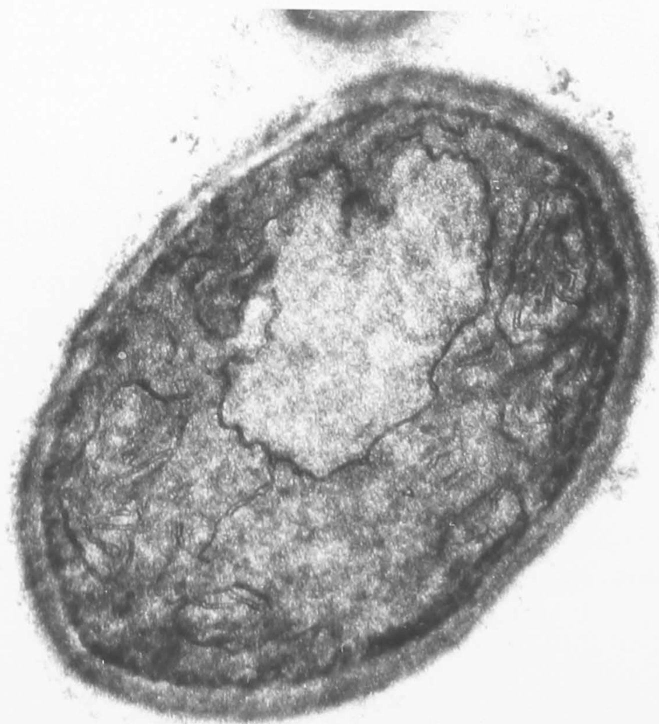
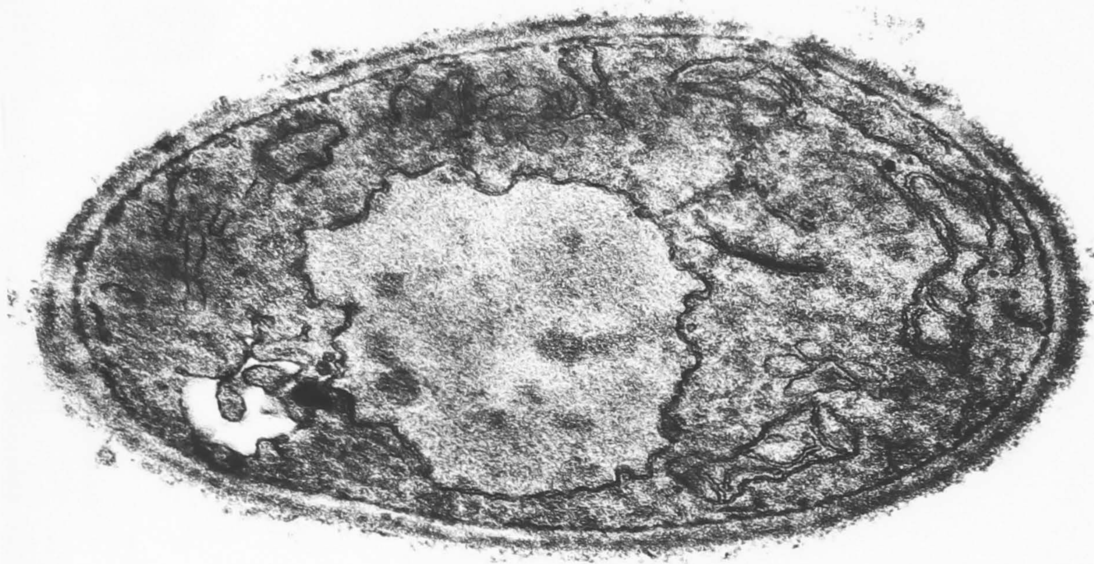




Figure 2-6. Electron micrographs of sections of cells of a CAP-treated (3 mg/ml) culture of *C. parapsilosis* at time T=11 hr.
An elongated mitochondrion can be seen in the upper cell section. Magnifications are 36000 (upper cell section) and 32000 (lower cell section).



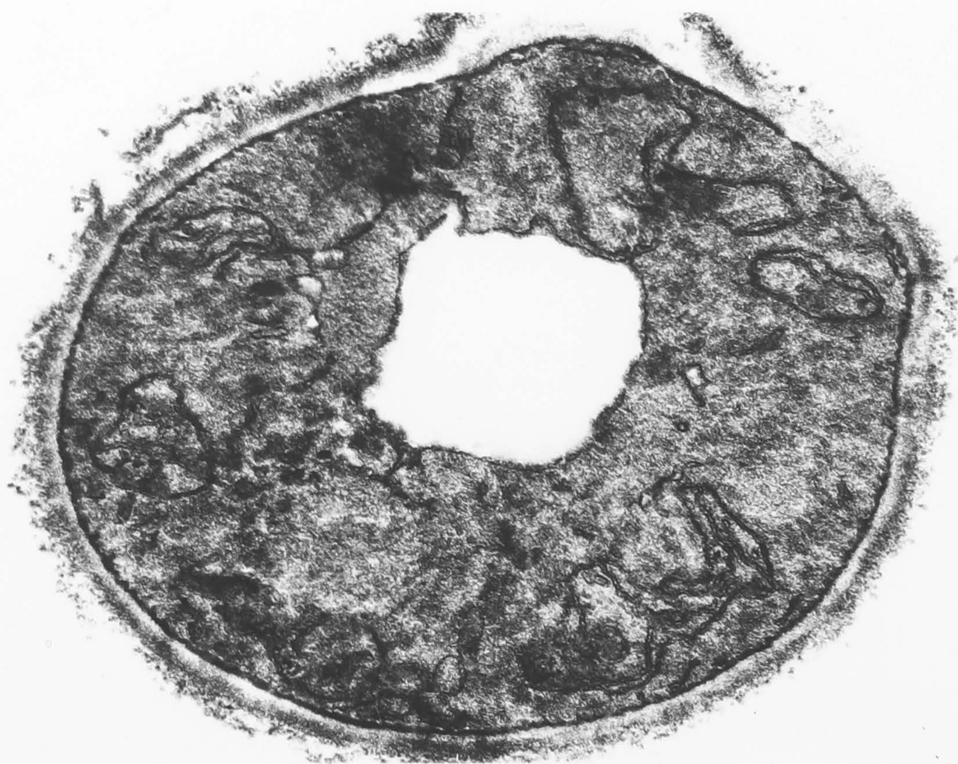
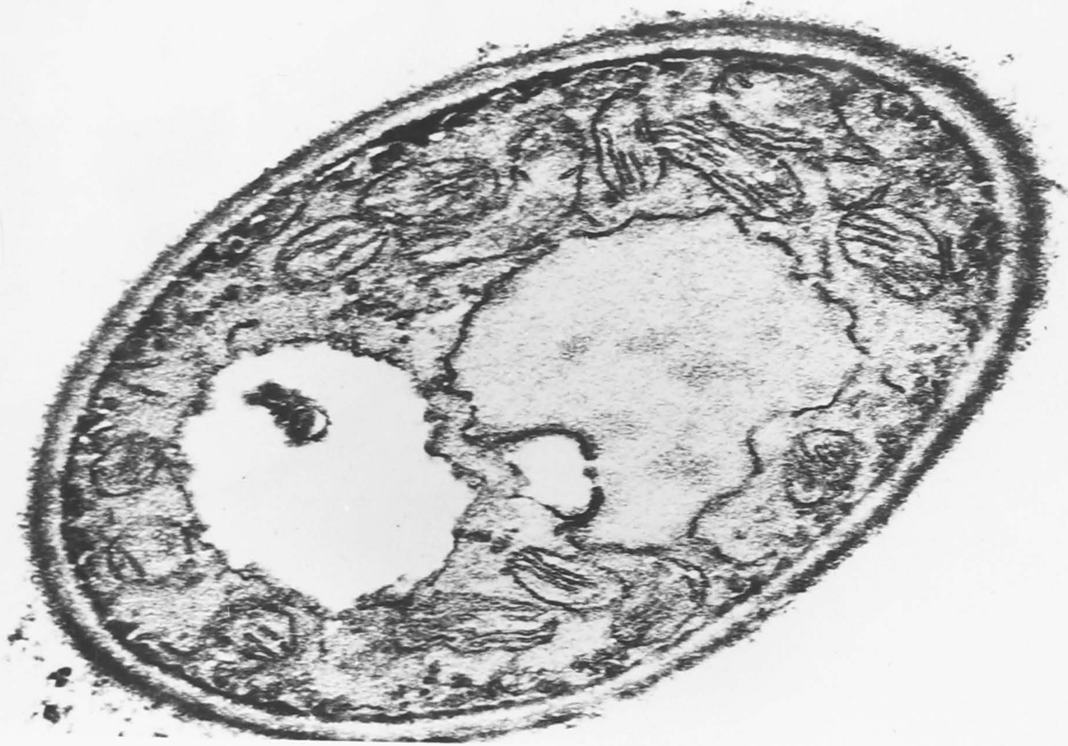


Figure 2-7. Electron micrographs of sections of cells of a normal culture of *C. parapsilosis* at time T=24 hr.
Mitochondria are more numerous than those in cells at T=11 hr. Magnifications of these cell sections are 30000 (upper cell section) and 22000 (lower cell section).



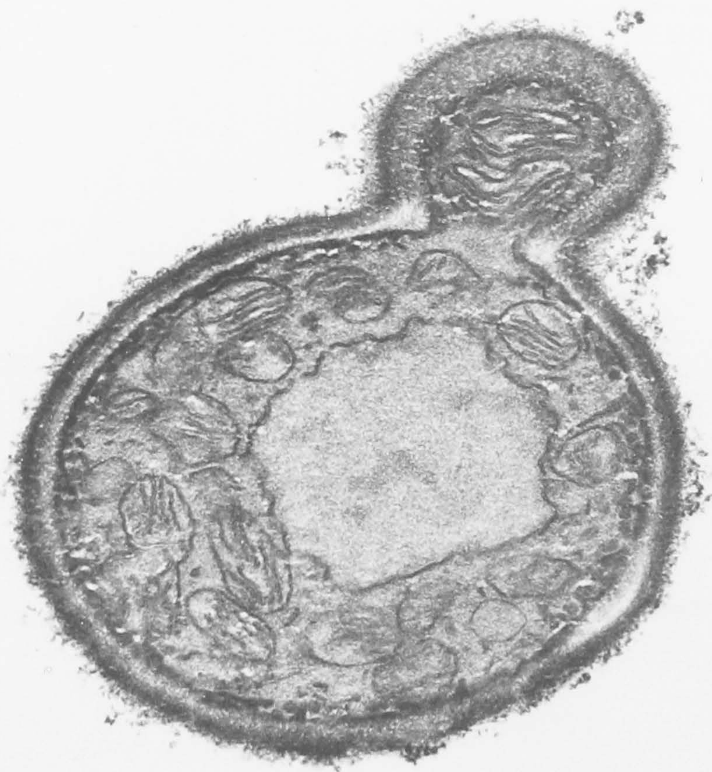


Figure 2-8. Electron micrographs of sections of cells of a CAP-treated (3 mg/ml) culture of *C. parapsilosis* at time T=24 hr.
Magnifications of these cell sections are 17000 (upper cell section) and 30000 (lower cell section).

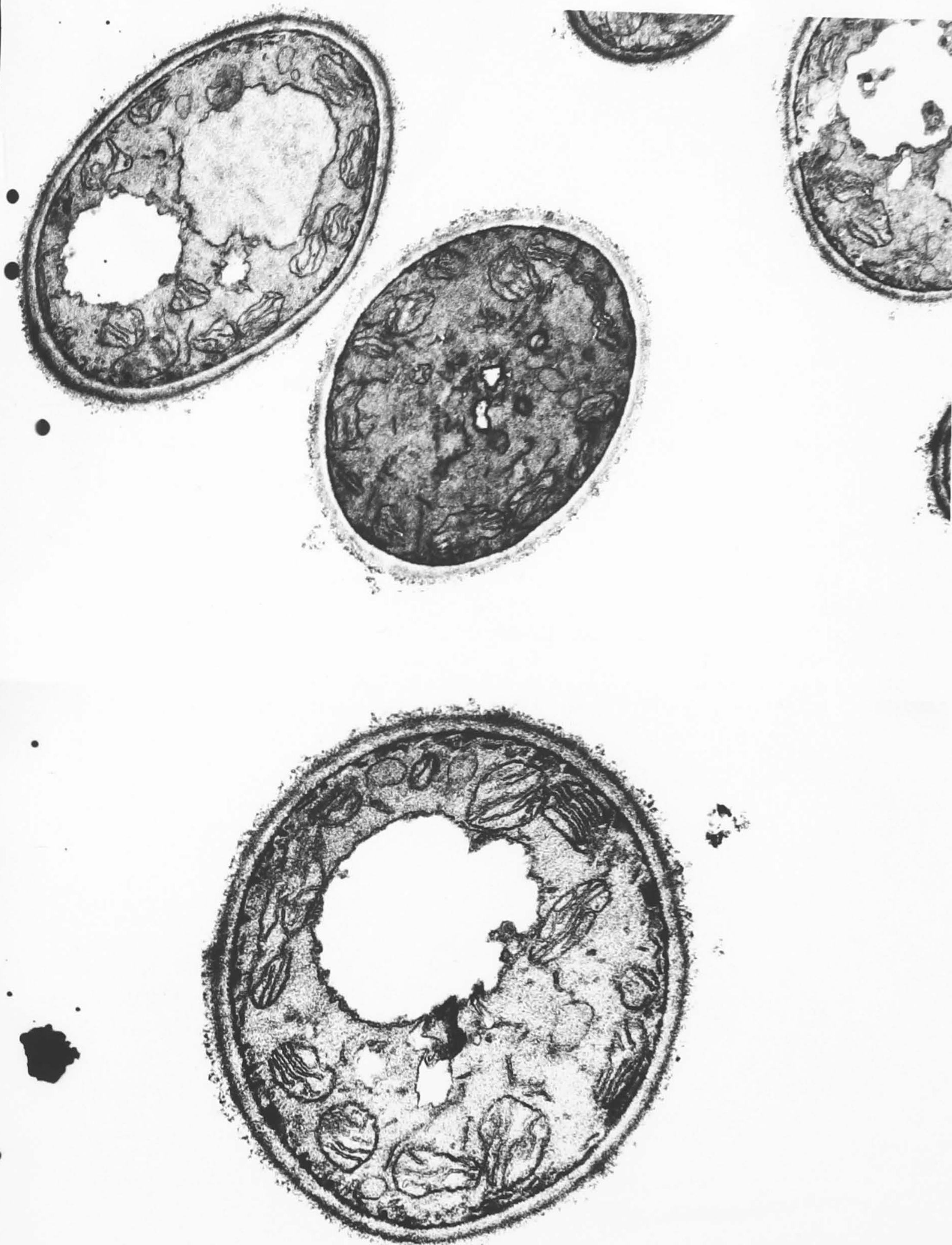
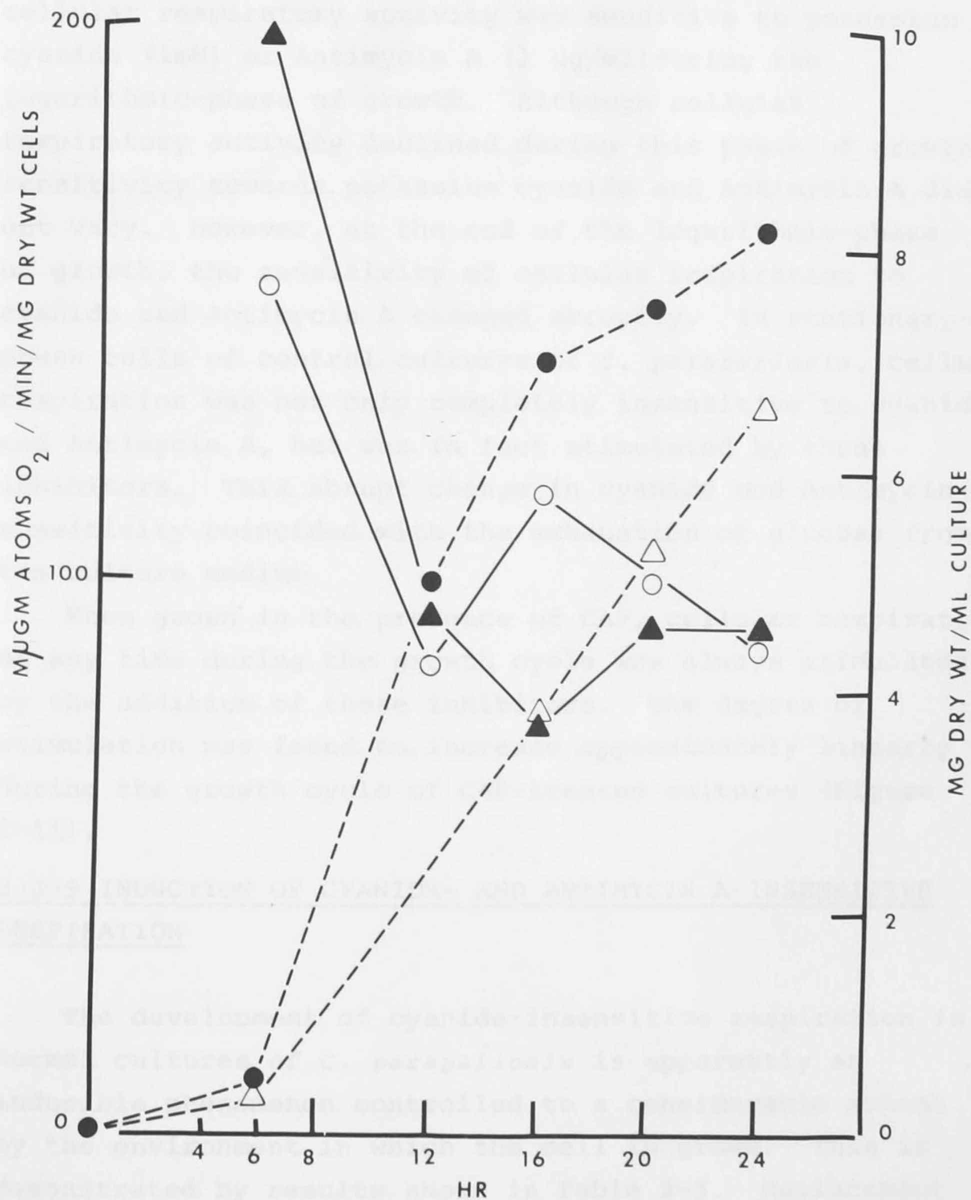


Figure 2-9. Cellular respiration during growth of cultures.

C. parapsilosis was cultured in *Saccharomyces* media containing 2.5% glucose (w/v) with and without addition of chloramphenicol (3 mg/ml). Growth (mg dry wt/ml culture) and respiratory activity (mμgm atoms O_2/mg dry wt cells) of cells were measured at different time during growth of these cultures. Cellular respiratory activities of normal and CAP-treated cultures are represented by ○——○ and ▲——▲ respectively. Growth of cells of normal and CAP-treated cultures are represented by ●------● and △------△ respectively.



200
100
0
MUGM ATOMS O_2 / MIN/MG DRY WT CELLS
10
8
6
4
2
0
MG DRY WT/ML CULTURE
4
6
8
12
16
20
24
HR

2-3-8 DEVELOPMENT OF CYANIDE- AND ANTIMYCIN A-INSENSITIVE RESPIRATION

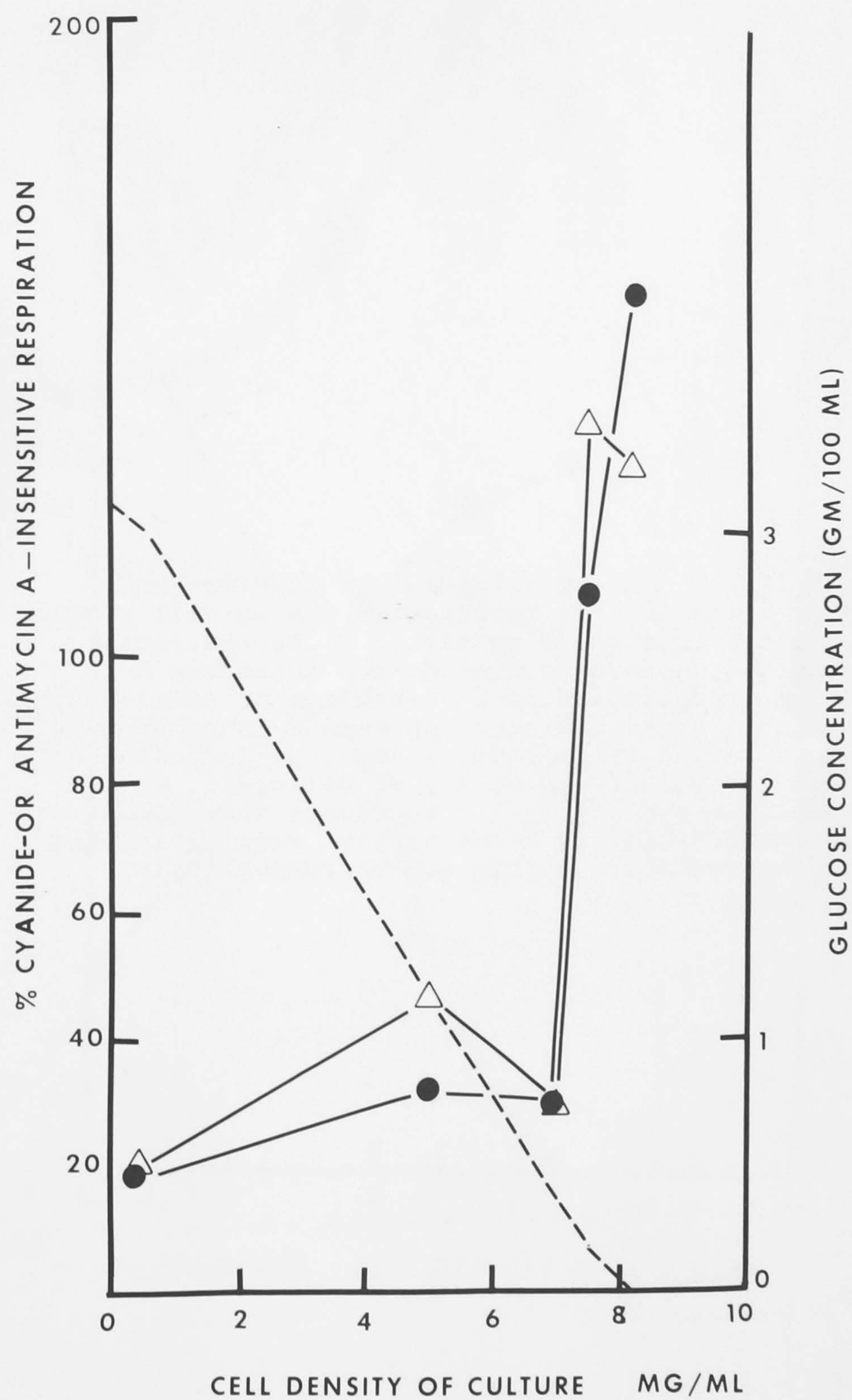
The sensitivity of the respiratory activity of *C. parapsilosis* towards inhibitors such as cyanide and Antimycin A showed another interesting feature (Figure 2-10). In the normal control culture, 70-80% of the cellular respiratory activity was sensitive to potassium cyanide (1mM) or Antimycin A (1 μg/ml)during the logarithmic-phase of growth. Although cellular respiratory activity declined during this phase of growth, sensitivity towards potassium cyanide and Antimycin A did not vary. However, at the end of the logarithmic-phase of growth, the sensitivity of cellular respiration to cyanide and Antimycin A changed abruptly. In stationary-phase cells of control cultures of *C. parapsilosis*, cellular respiration was not only completely insensitive to cyanide and Antimycin A, but was in fact stimulated by these inhibitors. This abrupt change in cyanide and Antimycin A sensitivity coincided with the exhaustion of glucose from the culture medium.

When grown in the presence of CAP, cellular respiration at any time during the growth cycle was always stimulated by the addition of these inhibitors. The degree of stimulation was found to increase approximately linearly during the growth cycle of CAP-treated cultures (Figure 2-11).

2-3-9 INDUCTION OF CYANIDE- AND ANTIMYCIN A-INSENSITIVE RESPIRATION

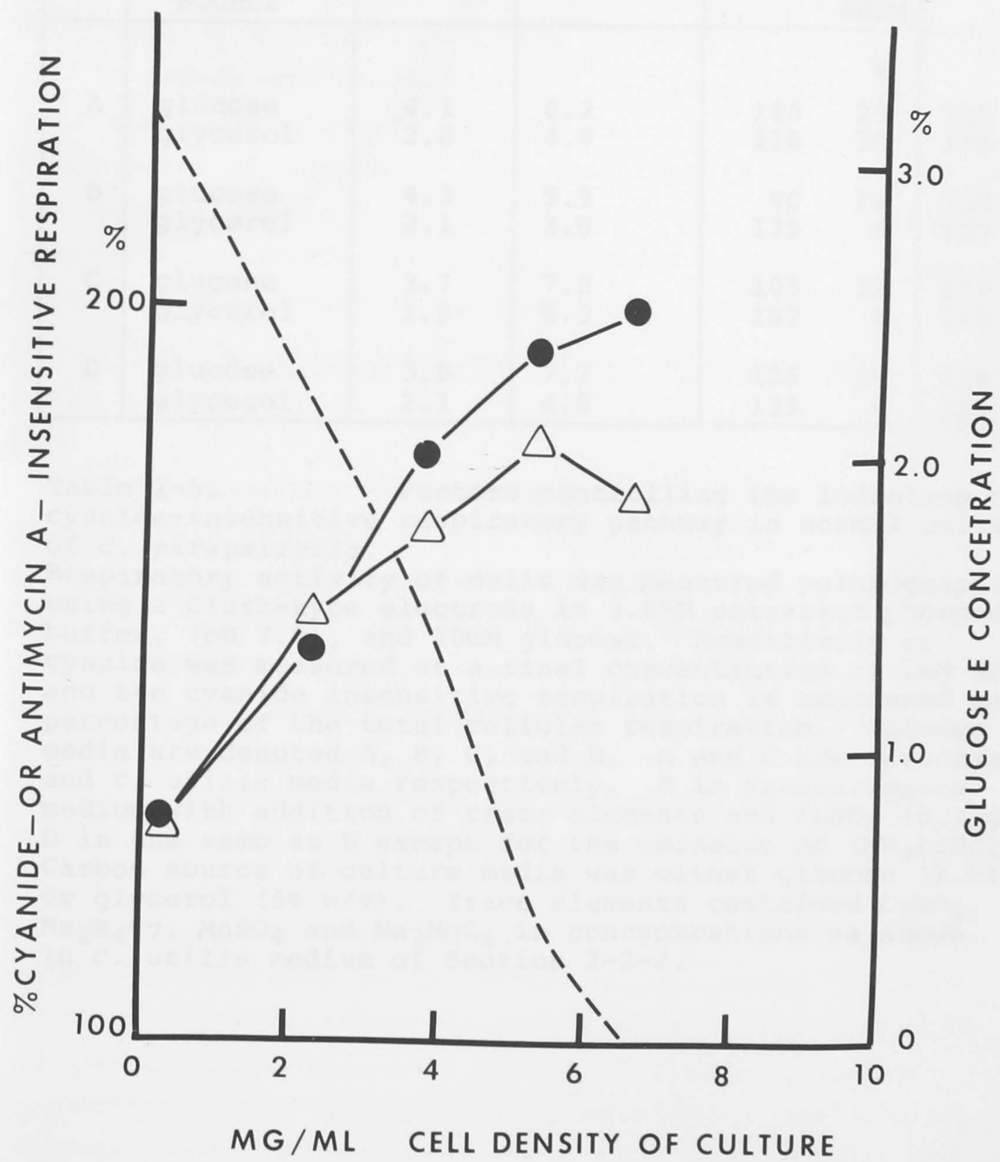
The development of cyanide-insensitive respiration in normal cultures of *C. parapsilosis* is apparently an inducible phenomenon controlled to a considerable extent by the environment in which the cell is grown. This is demonstrated by results shown in Table 2-5. Replacement of glucose in culture A and B by glycerol abolished the development of cyanide- insensitive respiration in stationary-phase cells. However, metabolites of glucose

Figure 2-10. Development of cyanide- and Antimycin A-insensitive respiration during growth of a normal culture of *C. parapsilosis.*
Left ordinate represents percentage cyanide- or Antimycin A-insensitive respiration; right ordinate represents concentration of glucose in the medium (gm/100ml). Abscissa indicates cell growth of the culture in mg dry wt cells / ml. Curves ●——●, △——△ and -------- represent development of cyanide-, and Antimycin A-insensitive respiration and glucose concentration respectively. Cyanide- and Antimycin A- sensitivities of cellular respiration were measured at 1 mM KCN and 1 μg/ml respectively.



200
100
80
60
40
20
% CYANIDE-OR ANTIMYCIN A—INSENSITIVE RESPIRATION
3
2
1
0
GLUCOSE CONCENTRATION (GM/100 ML)
0
2
4
6
8
10
CELL DENSITY OF CULTURE MG/ML

Figure 2-11. Development of cyanide- and Antimycin A-insensitive respiration during cell growth of a CAP-treated culture (3 mg/ml) of *C. parapsilosis*. Left ordinate represents cyanide- or Antimycin A-insensitive respiration as a percentage of total cellular respiration. Right ordinate represents concentration of glucose in medium in gm/100ml. Abscissa indicates cell growth of the culture in mg dry wt cells/ml. Curves ●——● , △——△ and ------ represent development of cyanide-, and Antimycin A-insensitive respiration and glucose concentration during growth respectively.


%CYANIDE–OR ANTIMYCIN A– INSENSITIVE RESPIRATION
%
200
100
%
3.0
2.0
1.0
0
GLUCOSE E CONCETRATION
0
2
4
6
8
10
MG/ML CELL DENSITY OF CULTURE

CULTURE MEDIUM		CELL GROWTH (mg dry wt cells/ml) AT		CELL RESPIRATION (mμgm atoms O_2/min/mg dry wt cells) AT			
	CARBON SOURCE	12 HR	24 HR	12 HR	+KCN %	24 HR	+KCN %
A	glucose	4.1	6.2	110	23	110	145
	glycerol	2.0	4.9	116	10	138	8
B	glucose	4.3	5.9	80	26	130	110
	glycerol	2.1	3.9	135	6	180	10
C	glucose	3.7	7.2	105	32	115	18
	glycerol	1.9	5.3	167	8	140	8
D	glucose	3.8	7.7	126	26	110	18
	glycerol	2.1	4.8	135	8	160	7

Table 2-5. Factors controlling the induction of cyanide-insensitive respiratory pathway in normal cultures of *C. parapsilosis*.
Respiratory activity of cells was measured polarographically using a Clark-type electrode in 0.05M potassium phosphate buffer, (pH 7.0), and 10mM glucose. Sensitivity to cyanide was measured at a final concentration of 1mM KCN, and the cyanide insensitive respiration is expressed as percentage of the total cellular respiration. Culture media are denoted A, B, C, and D. A and C are *Saccharomyces* and *C. utilis* media respectively. B is *Saccharomyces* medium with addition of trace elements and $ZnSO_4$ (0.1 gm/l). D is the same as B except for the omission of $(NH_4)_2SO_4$. Carbon source of culture media was either glucose (2.5% w/v) or glycerol (5% w/v). Trace elements contained $CuSO_4$, $Na_2B_4O_7$, $MnSO_4$ and Na_2MoO_4 in concentrations as shown in *C. utilis* medium of Section 2-2-2.

may not be the only factor(s) inducing the development of cyanide-insensitive respiration. By omitting ammonium sulphate in culture media (Cultures C and D), the development of cyanide-insensitive respiration could be abolished in the presence of glucose. It appeared that ammonium sulphate and metabolites of glucose were obligatory for the development of cyanide-insensitive respiration in stationary-phase cells of *C. parapsilosis*.

In CAP-treated cultures of *C. parapsilosis*, the presence or absence of ammonium sulphate and glucose had no apparent effect on the cyanide-insensitive respiration of the cell (Table 2-6a, Table 2-6b), nor on the phasic phenomenon of synthesis of cytochromes aa_3 and b suggesting therefore that these two phenomena are unrelated.

2-3-10 EFFECT OF CHLORAMPHENICOL ON MOLAR GROWTH YIELD IN *CANDIDA PARAPSILOSIS*

It has been demonstrated for *S. cerevisiae* that the growth yield in terms of cell mass generated per mole of glucose under conditions where glucose is the limiting nutrient can provide information concerning the intracellular mode of energy transformation. For instance, when the mode is both fermentative and oxidative, cell yield was in the order of 126 g per mole of glucose. When anaerobic fermentation was the only means of energy transformation, cell yield was about 26 g per mole of glucose (Kormancikova *et al.*, 1969).

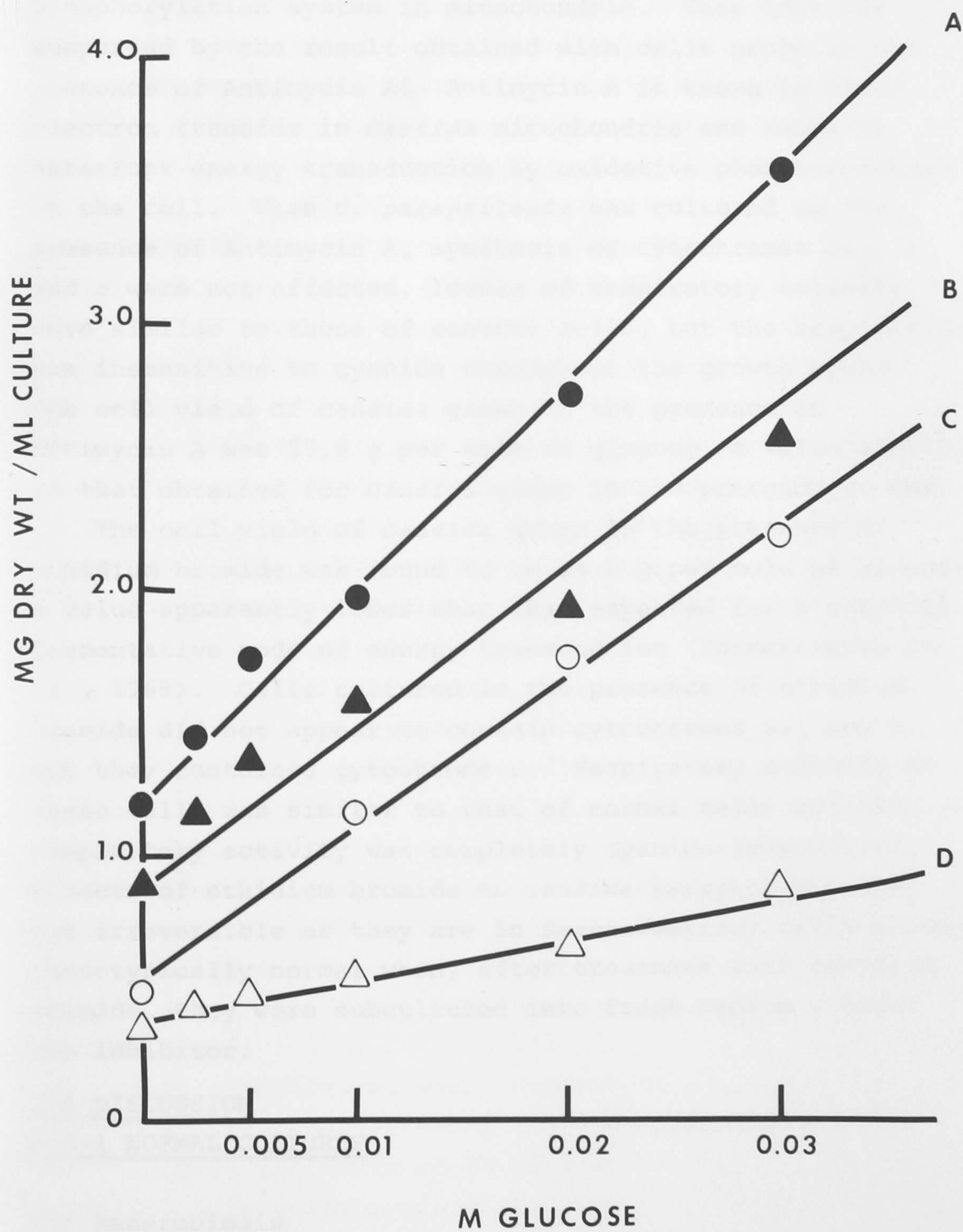
To assess the physiological significance of the altered respiratory pathway in chloramphenicol-treated cells in terms of the efficiency of transformation of energy from glucose to cell mass, cell yields at growth limiting concentrations of glucose were plotted as a function of glucose input. Figure 2-12 shows that cell yield bears a linear relationship to input of glucose for both normal and CAP-treated cultures of *C. parapsilosis*. Gradients from these plots gave cell yields for normal and CAP-treated cultures of 80 g per mole of glucose and 61.6 g per mole of glucose respectively. The efficiency of utilization of

	CELL TYPE	CELL GROWTH (mg dry wt cells/ml) AT		WHOLE CELL RESPIRATION (mμgm atoms O_2/min/mg dry wt cells) AT			
		10 HR	24 HR	10 HR	+KCN	24 HR	+KCN
A	*Saccharomyces* medium				%		%
	CONTROL						
	+2.5% glucose	2.5	9.2	137	23	78	123
	+2.5% glucose-$(NH_4)_2SO_4$	3.4	9.8	130	33	92	17
	CAP-TREATED						
	+2.5% glucose	1.0	6.6	165	118	95	179
	+2.5% glucose-$(NH_4)_2SO_4$	1.2	7.4	150	113	92	89
B	*C. utilis* medium						
	CONTROL						
	+2.5% glucose	4.3	8.6	128	15	77	10
	+5.0% glycerol	1.9	4.4	138	3	100	5
	CAP-TREATED						
	+2.5% glucose	1.7	7.1	156	95	70	115
	+5.0% glycerol	0.5	1.6	136	135	93	130

Table 2-6A. Effect of $(NH_4)_2SO_4$ on cyanide-insensitive respiration in CAP-treated cells.
Table 2-6B. Effect of carbon source on cyanide-insensitive respiration in CAP-treated cells.
Respiratory activities of cells were measured polarographically and sensitivities to cyanide were determined at the final concentration of 1 mM KCN. Respiratory activities of cells are expressed as mμgm atoms O_2/min/mg dry wt cells and cyanide sensitivity is expressed as percentage of the total cellular respiration.

Figure 2-12. Molar cell yield of *C. parapsilosis*. *C. parapsilosis* was cultured under conditions where glucose was the limiting nutrient. Final cell density of the cultures is shown as a function of initial glucose concentration. Gradients of lines of best fit indicate molar cell yields. Lines A, B, C and D represent yields for normal, CAP-treated (3 mg/ml), Antimycin A-treated (1 μg/ml) and ethidium bromide-treated (100 μg/ml) cultures. Molar cell yields of *C. parapsilosis* cultured under these conditions, calculated from gradients of the lines of best fit, are given in the insert.

CELL TYPE	MOLAR CELL YIELD (gm/mole)
Control	80
+CAP	61.6
+Antimycin A	57.6
+EB	14.8



A
B
C
D
4.0
3.0
2.0
1.0
0
MG DRY WT /ML CULTURE
0.005
0.01
0.02
0.03
M GLUCOSE

glucose in CAP-treated cells was thus almost 80% of that of the control, suggesting that the cyanide-insensitive respiratory pathway which exists in CAP-treated cells throughout the entire growth cycle might be coupled to generation of energy analogous to the classical oxidative phosphorylation system in mitochondria. This idea was supported by the result obtained with cells grown in the presence of Antimycin A. Antimycin A is known to block electron transfer in *Candida* mitochondria and hence to interrupt energy transduction by oxidative phosphorylation in the cell. When *C. parapsilosis* was cultured in the presence of Antimycin A, synthesis of cytochromes aa_3, *b* and *c* were not affected, levels of respiratory activity were similar to those of control cells, but the respiration was insensitive to cyanide throughout the growth cycle. The cell yield of *Candida* grown in the presence of Antimycin A was 57.6 g per mole of glucose, a value similar to that obtained for *Candida* grown in the presence of CAP.

The cell yield of *Candida* grown in the presence of ethidium bromide was found to be 14.8 g per mole of glucose, a value apparently lower than that expected for a complete fermentative mode of energy transduction (Kormancikova *et al.*, 1969). Cells cultured in the presence of ethidium bromide did not appear to contain cytochromes aa_3 and *b* but they contained cytochrome *c*. Respiratory activity of these cells was similar to that of normal cells but this respiratory activity was completely cyanide-insensitive. Effects of ethidium bromide on *Candida parapsilosis* were not irreversible as they are in *Saccharomyces*; cells became phenotypically normal when, after treatment with ethidium bromide, they were subcultured into fresh medium without the inhibitor.

2-4 DISCUSSION

2-4-1 NORMAL CULTURES

(i) Anaerobiosis

A fundamental question in biology is why some organisms (such as *Candida parapsilosis*) are obligate aerobes and why

others (such as *Saccharomyces cerevisiae*) are facultative anaerobes. In a strict sense, *S. cerevisiae* is also obligately aerobic because it will not grow prototrophically (i.e. in completely synthetic aqueous media containing glucose, mineral salts and biotin) under anaerobic conditions. However, vegetative propagation under anaerobic conditions can be sustained if synthetic media are supplemented with unsaturated fatty acids (UFA) and sterols (Andreasen and Stier, 1954). Biosynthesis of these growth factors requires molecular oxygen (Bloomfield and Bloch, 1958; Goldfine and Bloch, 1963). Thus *S. cerevisiae* is an obligate aerobe under stringent prototrophic conditions, and becomes a facultative anaerobe when UFA and sterols are provided.

C. parapsilosis can also propagate vegetatively in stringent prototrophic conditions provided oxygen is present. However, it will not propagate under anaerobic conditions in any enriched or supplemented medium so far devised. It is possible that unknown factors remain to be found that will permit vegetative propagation of *Candida* under anaerobic conditions. But it is also possible that no such hypothetical compound(s) exists to substitute for oxygen, and that *C. parapsilosis* is fundamentally different from *S. cerevisiae* in terms of its cellular requirement for oxidative activity. Inability to propagate anaerobically has also been reported for *Candida* by Kellerman *et al.* (1969), and for *Schizosaccharomyces pombe* by Heslot *et al.* (1970a). The oxygen requirement for growth of the latter organism could be relieved by another oxidant, potassium nitrate, thus enabling the organism to propagate anaerobically (Heslot *et al.*, 1970a). Potassium nitrate added to anaerobic cultures of *C. parapsilosis* failed to substitute for oxygen (Stewart, personal communication).

Thus, *C. parapsilosis* may require oxidative activity for survival, for instance to dispose of reducing equivalents generated intracellularly, or to support an obligate coupling of the oxidative system and cellular replication. It appears therefore that a careful analysis of the respiratory physiology of *Candida parapsilosis* may throw

some light on this fundamental problem.

(ii) Catabolite repression

In contrast to *S. cerevisiae,* catabolite or glucose repression of synthesis of the electron transfer chain did not appear to operate in *C. parapsilosis.* Also, results on the synthesis of cytochromes (measured by absorption spectroscopy and enzyme assays, electron microscopy of cells and cellular respiratory activity) demonstrated that, in a normal culture, synthesis of cytochromes did not respond to variations in external concentrations of glucose. *S. cerevisiae,* on the other hand, shows many biochemical and ultrastructural differences between glucose-repressed and derepressed cultures, most of which have been extensively documented (see section 1-3-2).

(iii) Cytology

In glucose-rich (logarithmic phase) and glucose-depleted (stationary phase) cultures of *C. parapsilosis,* cells were similar in ultrastructure. Mitochondria with well-defined double membranes and distinct cristae were present, though apparently in greater number in stationary phase cells. Apart from a small increase in the number of cristae per mitochondrion in stationary phase (glucose-depleted) cells, mitochondria in cells of glucose-rich and glucose-depleted phase of a growth cycle were indistinguishable. Similar results were reported by Kellerman *et al.* (1969) using the same organism. Absence of glucose repression has also been reported to be a general phenomenon of many petite-negative, obligate aerobes (Bulder, 1964 a, b; De Deken, 1966; McClary and Bowers, 1968). Results presented here are in agreement with these earlier reports.

(iv) Respiratory activity

Oxidative activity in *C. parapsilosis* declined abruptly to a constant level during the logarithmic phase of growth while external glucose concentration was still high and remained constant in stationary phase of growth. This contrasts with the development of cyanide- and Antimycin A-sensitive oxidative activity in *S. cerevisiae* during a normal growth cycle (Wallace and Linnane, 1964). However,

while in *C. parapsilosis* the oxidative activity remained constant during subsequent growth, an alternate oxidative pathway, insensitive to both cyanide and Antimycin A, developed in the cell. Development of this cyanide-insensitive pathway required the presence of $(NH_4)_2SO_4$ and metabolites of glucose, and occurred at a specific phase in the growth cycle, namely the stationary phase. The nature and mechanism of this rapid development of the alternate pathway, and the part played by $(NH_4)_2SO_4$ and glucose remains to be determined. It would be of interest to determine the location of this pathway, for instance whether it is also localized in the mitochondrion or other cellular organelles such as peroxisomes.

Heslot *et al.* (1970a) have observed the presence of cyanide- and Antimycin A-insensitive respiration in normal cultures of *Schizosaccharomyces pombe* when cells were grown in glucose but no such respiration was detected in cells grown in glycerol. A high percentage of cyanide-insensitive respiration in normal cells was reported by Kellerman *et al.* (1969) using *Candida,* and similar observations on *Pythium ultimum* have been made by Smith and Marchant (1968). Published data cited refer to one specific point during the growth cycle of the culture and hence do not provide information as to the regulation or development of this alternative oxidative pathway. It would be of great interest to know if development of the pathway insensitive to cyanide and Antimycin A is a general phenomenon of obligate aerobes.

2-4-2 EFFECT OF CAP AND ER

(i) Growth

Saccharomyces cerevisiae obviously is well adapted, since the rate of growth is unaffected in the absence of mitochondrial respiratory activity so long as an ample source of fermentable substrate is available (Linnane *et al.,* 1968). Of course, in the absence of any available fermentable substrate, cell growth is completely arrested when activity of the respiratory pathway is precluded.

For example in the presence of chloramphenicol growth continues only for as long as fermentable substrate is available (Linnane *et al.*, 1968).

Insufficiency of the glycolytic capacity of *C. parapsilosis* to satisfy minimum cellular demands for the support of cellular processes might account for its failure to grow anaerobically. In the presence of chloramphenicol, the rate of growth was lowered significantly, but the molar cell yield was only slightly affected. Thus the efficiency of energy conservation and transformation in the chloramphenicol-treated and normal cells appear very similar. The absence of normal mitochondrial respiratory function for at least part of the growth period thus did not greatly affect the overall transformation of substrates into cell mass. The reason why chloramphenicol or erythromycin affected the absolute rate of growth in logarithmic-phase might be that the rate at which substrate is taken up or transformed to usable forms of energy in the cell is affected by antibiotics, while the overall efficiency of transformation is not.

Similar results have been reported by Kellerman *et al.* (1969). They found that while chloramphenicol (at 4 mg/ml) resulted in a prolongation of the generation time in the logarithmic phase from the normal 1.4 hr to 2.8 hr, the final cell yield was unaltered. In contrast, Linnane *et al.* (1968) reported that "the growth of the obligate aerobe *Candida parapsilosis* in liquid medium containing 5% glucose was arrested by chloramphenicol. The cell yield from a 16 hr culture decreased by about 50% in the presence of 1 mg/ml of chloramphenicol and at concentration of 4 mg/ml of chloramphenicol growth is almost completely inhibited". No explanation can be offered for the discrepancy between results of Linnane *et al.* (1968) on one hand and the agreement between results of Kellerman *et al.* (1969) and my results on the other.

Chloramphenicol has also been observed to depress the rate of growth as well as the final cell yield of *Rhodotorula glutinis* on a glucose medium. In glycerol medium, however,

chloramphenicol only affected the rate but not the final cell yield (Smith and Marchant, 1968). However, when chloramphenicol was added to a culture of *R. glutinis* in glucose or glycerol medium the effect on rate of growth was not as marked as when present in the medium from the start (Smith and Marchant, 1968). In the obligate aerobe, *Pythium ultimum,* chloramphenicol inhibited both the rate of growth and final cell yield in liquid culture; on a solid medium the effect of chloramphenicol on the rate of linear growth was far less than the effect in liquid culture. But the chloramphenicol-treated culture on solid medium had a far lower density of hyphae and less aerial mycelia than the control (Marchant and Smith, 1968).

(ii) Cytology

Apart from a suppression by chloramphenicol of the formation of cristae in logarithmic phase of growth, the cytology of CAP-treated cells of *C. parapsilosis* was similar to that of normal cells throughout growth. Kellerman *et al.* (1969) and Linnane *et al.* (1968) reported that cells of *C. parapsilosis* contained very elongated mitochondria whose outer membranes appeared normal, but whose cristae were of an abnormal, fragmented character present in much smaller amount than normal. Unfortunately, evidence presented in these two reports is of single cell sections at one particular point during the growth cycle; no numerical analysis was made. As shown in section 2-3-6 elongated mitochondria were observed in logarithmic and stationary phase cells of both normal and CAP-treated cultures of *C. parapsilosis,* but these elongated mitochondria appeared normal with respect to the state of cristae and double membrane structure and were observed in cells which also contained round and oval shaped mitochondria.

Mitochondria in hyphae of *Pythium ultimum* grown in the presence of chloramphenicol resembled mitochondria in normal hyphae, but were devoid of stalked particles (Marchant and Smith, 1968). On the other hand, chloramphenicol produced a progressive deterioration in the organization of mitochondria in *Rhodotorula glutinis.*

At a sufficiently high concentration of chloramphenicol, gross distortion of the cristae and of the overall mitochondrial profile was apparent (Smith and Marchant, 1968). It should be re-emphasized that evidence presented in these reports was of typical cell sections without accompanying numerical analysis.

(iii) Respiratory activity

It is possible that the respiratory pathway which developed in response to CAP treatment (insensitive to cyanide and Antimycin A) was the same as the one found in stationary phase cells of a normal culture of *Candida* and is a cellular defence mechanism to ensure survival when the synthesis of the electron transfer chain is inhibited. Linnane *et al.* (1968) have observed also that cells of *C. parapsilosis* cultured in the presence of chloramphenicol for 16 hr in 5% glucose medium had respiratory activity as high as the normal. It is not known however, if the respiratory activity was sensitive to cyanide or Antimycin A. On the other hand, Kellerman *et al.* (1969) reported that CAP slightly decreased the respiratory activity of cells of *C. parapsilosis* as compared with normal cells, and found that the respiratory activity in chloramphenicol-treated cells is largely insensitive to cyanide. In the case of *Rhodotorula glutinis,* cells cultured in the presence of chloramphenicol had almost normal respiratory activity which was however completely insensitive to inhibition by Antimycin A (Smith and Marchant, 1968). Similar results were also obtained for *Pythium ultimum* (Smith and Marchant, 1968).

(iv) Synthesis of cytochromes

It is now well-known that in *S. cerevisiae* chloramphenicol inhibits the synthesis of cytochromes aa_3 and *b* but not *c*, an effect which is persistent throughout the entire growth cycle of the culture. Experimental data presented in this chapter show that synthesis of cytochromes aa_3 and *b* in *C. parapsilosis* is inhibited by CAP and ER only when cells are actively-dividing i.e. in logarithmic phase of the growth cycle. Synthesis of cytochromes was not inhibited in

cells not actively dividing i.e. in stationary phase.

Inhibition of synthesis of cytochomes aa_3 and b by chloramphenicol was reported by Kellerman *et al.* (1969) and Linnane *et al.* (1968) for *C. parapsilosis,* and for *Pythium ultimum* by Marchant and Smith (1968). However the effect of chloramphenicol on the synthesis of cytochromes has not been examined at any other phase of a growth cycle of these organisms and the results reported presumably represent an effect of chloramphenicol at one particular time in a growth cycle.

The phasic phenomenon observed in *Candida parapsilosis* involved an apparent change of cellular sensitivity towards inhibition by CAP and ER. Many hypotheses of the nature and cause of this phasic response could be put forward on the basis of the data presented in this chapter. Experimental tests of such hypotheses are presented in the next chapter.

In summary, a comparative study of *Candida parapsilosis* and *Saccharomyces cerevisiae* provided evidence of differences between these two organisms. These differences were (1) inability of *C. parapsilosis* to grow under anaerobic conditions on any supplemented medium so far devised; (2) apparent absence of glucose repression on the synthesis of the electron transfer chain in *Candida*; (3) development of a cyanide- and Antimycin A-insensitive pathway in *Candida* which might be activated by $(NH_4)_2SO_4$ and glucose in normal cells in stationary phase of growth, and may be induced by antibiotics such as CAP; and (4) transient inhibition of synthesis of cytochromes aa_3 and b in *Candida* by CAP and ER in logarithmic phase cells; stationary phase cells synthesised near normal amounts of these cytochromes in the presence of the inhibitors.

CHAPTER 3

A COMPARATIVE STUDY OF THE RESPIRATORY PHYSIOLOGY OF *C. PARAPSILOSIS* AND *S. CEREVISIAE* (II) CAUSE AND NATURE OF THE PHASIC PHENOMENON.

CHAPTER 3

3-1 INTRODUCTION

In the preceding chapter, a phasic phenomenon was described for *Candida parapsilosis*. When *C. parapsilosis* was cultured in the presence of inhibitors of the mitochondrial protein synthesising system (chloramphenicol and erythromycin), synthesis of cytochromes aa_3 and b was inhibited in logarithmic phase but not in stationary phase of growth. The apparent loss of sensitivity towards chloramphenicol and erythromycin in stationary phase cells prompted an investigation into the cause and nature of this phenomenon. This chapter describes experimental results which may be interpreted to mean that the phasic phenomenon is, in effect, an aspect of metabolic regulation controlling cellular synthesis of enzymes such as cytochromes aa_3 and b.

3-2 EXPERIMENTAL

Most of the procedures and methods used in experiments described in this chapter are outlined in Chapter 2. Additional techniques and procedures are as follows.

3-2-1 SYNTHETIC MEDIUM

The synthetic medium was composed of a carbon source, mineral salts and vitamins in the following proportion:-

	(per litre)
Glucose	25 gm
$(NH_4)_2SO_4$	1.2 gm
NaCl	0.5 gm
$CaCl_2$	0.1 gm
$MgSO_4.7H_20$	2.0 gm
KH_2PO_4	1.0 gm
$FeCl_3$	0.0005 gm
Trace elements	5 ml of stock solution
Vitamins	1 ml of vitamin cocktail

Stock solution of trace elements was made up of:

	(per litre)
$CuSO_4.5H_2O$	40 mg
KI	100 mg
$ZnSO_4.7H_2O$	100 mg
$Na_2MoO_4.2H_2O$	100 mg
$Na_2B_4O_7.10H_2O$	100 mg
$MnSO_4.4H_2O$	200 mg

Vitamin cocktail was made up of:

	(per litre)
Calcium pantothenate	1000 mg
Thiamine	1000 mg
Inositol	2000 mg
Pyridoxine hydrochloride	1000 mg
Nicotinic acid	500 mg
Biotin	200 mg

3-2-2 FORMATION OF YEAST PROTOPLASTS AND ISOLATION OF MITOCHONDRIA

Yeast protoplasts were prepared by digestion of the cell wall with snail-gut enzymes. Thoroughly washed cells were suspended in two volumes of pre-incubation buffer (mercaptoethanol 0.1M, sodium EDTA 0.02M, and Tris-HCl 0.02M pH 9.3) and incubated aerobically with regular agitation at 37°C for 30 min. Cells were then washed three times by resuspending in sorbitol phosphate buffer (sorbitol 0.9M, potassium phosphate 0.02M, pH5.8, citric acid 1.37 gm per litre, and sodium EDTA 0.001M, pH5.8) and collected by centrifugation. Following suspension of packed cells in 1.3 volumes of sorbitol phosphate buffer, a preparation of snail-gut enzymes (0.25 volume) was added to the cell suspension. The mixture was incubated at 37°C and the formation of protoplasts was followed by the rate of decrease in optical density at 640 mμ of a 2000-fold dilution of the digestion mixture with water. When the optical density had reached 15% of the initial value, protoplasts were collected by centrifugation at 5000 rpm for 5 min (Sorvall, GSA). Snail-gut enzymes were removed

by washing protoplasts three times with sorbitol phosphate buffer at $0^{o}C$. Washed protoplasts were resuspended in one fifth volume of sorbitol phosphate buffer and then two volumes of autoclaved distilled water were added to lyse them. The suspension was dispersed in a Potter-Elvehjem homogenizer to facilitate disruption of protoplasts, and the pH of the lysate was immediately adjusted to 7.0 with KOH(1M). Unbroken protoplasts and undigested cells were removed by centrifugation at 3000 rpm for 10 min (GSA, Sorvall) three times. The resultant supernatant was then centrifuged at 9000 rpm for 20 min (Sorvall, SS34) to sediment mitochondria. The mitochondrial fraction was then washed twice by resuspension in mitochondrial resuspension buffer (sorbitol 0.5M, Tris HCl 0.01M, pH 7.4, sodium EDTA 0.5 mM).

3-2-3 *IN VITRO* AMINO ACID INCORPORATION BY ISOLATED MITOCHONDRIAL FRACTIONS

Amino acid incorporation was carried out in a buffered system composed of the following. The method is essentially that of Lamb *et al.* (1968).

Buffer (KCl 0.2M, $MgCl_2$ 0.026M, Tris HCl 0.07M, pH 7.4)	0.5 ml
ATP-regenerating system	0.1 ml
Antibiotics (or H_2O)	0.1 ml
H_2O	0.1 ml
Mitochondrial suspension (containing approximately 1 mg protein)	0.2 ml
L-leucine-2-^{14}C (10 µCi/ml)	0.02 ml

The ATP-regenerating system consisted of ATP (potassium salt) 30 mg, PEP (dipotassium salt) 50 mg, pyruvate kinase (rabbit skeletal muscle, type II, Sigma Co; one mg converts approximately 350 µmoles PEP to pyruvate per min at pH 7.6, $37^{o}C$) 0.04 ml and oligomycin 0.5 mg in 3 ml of solution. 0.1 ml of this stock solution was used for each assay. Water was used to replace the ATP-regenerating system as appropriate.

The reaction was carried out at $30^{o}C$ with regular agitation and terminated by addition of 5 ml of 7% TCA solution containing unlabelled D, L-leucine (5mM).

The precipitate was washed twice with 7% TCA, once with absolute ethanol, once with acetone, defatted in ethanol-ether (1:1 v/v) at 65°C for 15 min, cooled and washed with ether. The residue was resuspended in 1 ml of distilled water, dispersed in 10 ml of dioxane-naphthalene-PPO (Beckman Instruction Manual, PPO 5 gm per litre, naphthalene 100 gm per litre, dioxane balance of litre) and counted in a Beckman LS-250 scintillometer.

3-2-4 UPTAKE OF CHLORAMPHENICOL BY CELLS

Cells were removed from cultures and washed five times with distilled water at 35°C. In the case of cells from CAP-treated cultures, this treatment was found in preliminary experiments to be effective in removing 80% of CAP bound to cells. Washed cells were then resuspended in *Saccharomyces* medium containing 2.5% glucose and C^{14}-CAP of constant specific radioactivity at different concentrations of CAP. Incubation was carried out at 30°C for 60 min. Cells were then cooled in ice, removed from the medium by centrifugation and washed with large volumes of cold (0°C) distilled water five times. Washed cells were then resuspended in 1 ml of distilled water and transferred quantitatively into scintillation vials containing 10 ml of Cabosil gel in scintillation fluid. After mixing, vials were assayed for radioactivity. Efficiency of counting under these conditions was approximately 95%.

3-2-5 DETERMINATION OF CHLORAMPHENICOL IN CULTURE

(i) Method of Levine and Fischback (1951)

5 ml of phosphate buffer (0.2M potassium phosphate, pH 6.0) was added to 2 ml of appropriately-diluted, cell-free culture medium. The resultant solution was then extracted twice with 25 ml of chloroform ethylacetate (2:1 v/v). Extracts were pooled and filtered through filter paper. The filtrate was then evaporated to dryness in a steam bath under a current of air. The residue was dissolved in 3 ml of NaOH (0.1M), warmed, and shaken. Approximately 25 mg of sodium dithionite was added and the

mixture was incubated at room temperature for 15 min. 0.5 ml of aqueous $NaNO_2$ (5% w/v) was added, followed by 10 drops of concentrated HCl and allowed to stand at room temperature for 3 min. 1 ml of sulphamic acid (5% w/v) was then added to initiate the diazotization, and the reaction was allowed to proceed under vacuum. Then 0.5 ml of N-1-naphthyl ethylene diamine dihydrochloride (0.5% w/v) was added to complete diazotization. A pink colour developed on standing for 2 hr and was read against the reagent blank at 558mμ. The standard curve was linear in the range of 2-20 μg CAP.

(ii) Method of Hughes and Diamond (1964)

2.0 ml of potassium phosphate buffer (0.1M, pH 7.0) was added to 1.0 ml of sample solution. Water was used in the reagent blank tube. 3.0 ml of isoamyl acetate was added, the tube was stoppered, shaken well for 10 min at room temperature and then centrifuged. 2 ml of the resultant supernatant solvent was mixed with 1 ml of NaOH (1.5M) and 1 ml of aqueous isonicotinic acid hydrazide (3% w/v). The mixture was incubated in a stoppered tube at 30°C for 45 min. The stoppered tube was shaken well at regular intervals. At the end of the incubation period the yellow coloured underlayer was aspirated and the absorbance read at 430 mμ against the reagent blank. The standard curve was linear up to 50 μg of CAP.

3-2-6 *IN VIVO* ASSAY OF MITOCHONDRIAL PROTEIN SYNTHESIS

Cells were removed from cultures, washed three times with large volumes of distilled water at 35°C and once with glucose-phosphate buffer (potassium phosphate, 0.05M pH 7.0, glucose 0.1% w/v). Washed cells were resuspended in glucose-phosphate buffer at 10 mg dry wt/ml. Antibiotics were added to aliquots of the cell suspension at concentrations specified. Cell suspensions were equilibrated with antibiotics for 15 min at 29°C. 10 μCi of L-leucine-4, 5-^{3}H (34.1 mCi/μmoles) was added per 100 ml of cell suspension and incorporation of the label was allowed to continue for 15 min. Incorporation was terminated by adding excess (5000-fold dilution) nonradioactive leucine, and

immediately cooling cell suspensions. Cells were immediately harvested by centrifugation at $2^{o}C$, and were washed once with cold STE (sorbitol 0.5M, Tris HCl 0.01M pH 7.4, sodium EDTA 0.5mM, pH 7.4), and resuspended in one volume of STE (mitochondrial resuspension buffer) per volume of packed cells. One volume of glass beads (0.45mm-0.5mm diameter) per volume of cell suspension was added, and cells were broken in a Braun MSK homogeniser operated at full speed for 25 sec with carbon dioxide as coolant. The brei was decanted and glass beads were rinsed with one volume of STE. The brei and washings were pooled and centrifuged twice at 3000 rpm (Sorvall, GSA) for 10 min to remove cell debris and nuclei. The resultant supernatant was denoted cell-free homogenate. The cell-free homogenate was centrifuged at 9000 rpm (Sorvall, SS34) for 20 min to sediment mitochondria. The supernatant was denoted post-mitochondrial supernatant. The mitochondrial fraction was washed twice with STE. Aliquots of subcellular fractions were precipitated with 7% TCA and processed for radioactivity counting as described above (Section 3-2-3).

3-2-7 WHOLE CELL INCORPORATION OF C^{14}-LEUCINE INTO PROTEIN

15 min after addition of antibiotics to cultures at 14 hr, 1 ml aliquots of cell cultures were removed and mixed with 0.2 ml of C^{14}-leucine (0.5 μCi/ml, 311 μCi/μmole) in centrifuge tubes. Incorporation of labelled leucine was allowed to proceed for 15 min with agitation at $29^{o}C$. To terminate the incorporation, 6 ml of 7% TCA containing D, L-leucine (1 mg/ml) was added to each tube. For zero-time incorporation, TCA was added immediately prior to addition of C^{14}-leucine. For assay of incorporated radioactivity, cells in 7% TCA were heated at $90^{o}C$ for 15 min, dried with acetone, defatted with ethanol-ether (2:1 v/v) and dried with ether. The dried residue of cells was taken up in 0.5 ml of water and quantitatively transferred into a counting vial containing 10 ml of Cabosil gel in dioxane-based scintillation fluid.

3-3 RESULTS

3-3-1 REVERSIBILITY AND ENVIRONMENTAL CONTROL OF THE PHASIC PHENOMENON

Data bearing on the nature of the phasic phenomenon are shown in Figures 3-1, 3-2 and 3-3.

Figure 3-1 demonstrates the reversibility of the apparent change in the sensitivity of cells towards inhibition by CAP of the synthesis of cytochromes aa_3 and *b*. When stationary phase cells of a CAP-treated culture, which had resynthesised cytochromes aa_3 and *b*, were subcultured into fresh medium with no inhibitors added, they grew and synthesised cytochromes aa_3, *b* and *c* at rates identical to fresh cultures inoculated with untreated cells. When resultant stationary phase cells cultured in the absence of CAP were again subcultured into fresh medium with chloramphenicol added, the culture was found to exhibit the phasic phenomenon (i.e. synthesis of cytochromes aa_3, and *b* was inhibited at logarithmic-phase of growth but not at stationary phase). The phasic phenomenon is thus reversible and unlikely to be due to selection and accumulation of antibiotic-resistant mutants during growth in the presence of antibiotics. This was also supported by experimental results shown in Figure 3-2. When cells taken from the logarithmic or from the stationary phase of growth of a CAP-treated culture were subcultured into fresh medium with CAP, both subcultures responded identically to the antibiotic. If selection and accumulation of antibiotic-resistant mutants occurred during growth in the presence of antibiotics, stationary phase cells would have consisted to a high proportion, if not entirely, of antibiotic-resistant mutants. On sub-culturing stationary phase cells of a CAP-treated culture into fresh medium containing CAP, synthesis of cytochromes aa_3 and *b* would occur early in the growth cycle and exhibit no phasic response towards inhibition by the antibiotic. Experimentally, this was not the case (Figure 3-2). It is thus highly unlikely that the phasic phenomenon is genetically based, but rather it is due to changes in the culture environment.

Figure 3-1. Reversibility of the phasic phenomenon. Cells of a *CAP-treated culture* grown for 24 hr (stationary phase) were subcultured into antibiotic-free fresh medium *(normal culture)* and allowed to grow to time T=24 hr. These cells were then again subcultured into fresh medium containing chloramphenicol (3 mg/ml) *(CAP-treated)*. Cells of the primary culture, and the first and second subcultures were examined for cytochrome contents at time T=12 and T=24 hr. The standard initial inoculum of 0.03 mg/ml was used for each culture. Culture medium used was 2.5% glucose *Saccharomyces* medium. For clarity of presentation, absorption spectra of cells were not presented but summarized. aa_3, b and c means presence of cytochromes aa_3, *b* and *c* as represented by the absolute cytochrome absorption spectrum of cells of a CAP-treated culture at T=24 hr (Figure 2-3C) and c means absence of cytochromes aa_3 and *b* as represented by cells of a CAP-treated culture at time T=10 hr (Figure 2-3C).

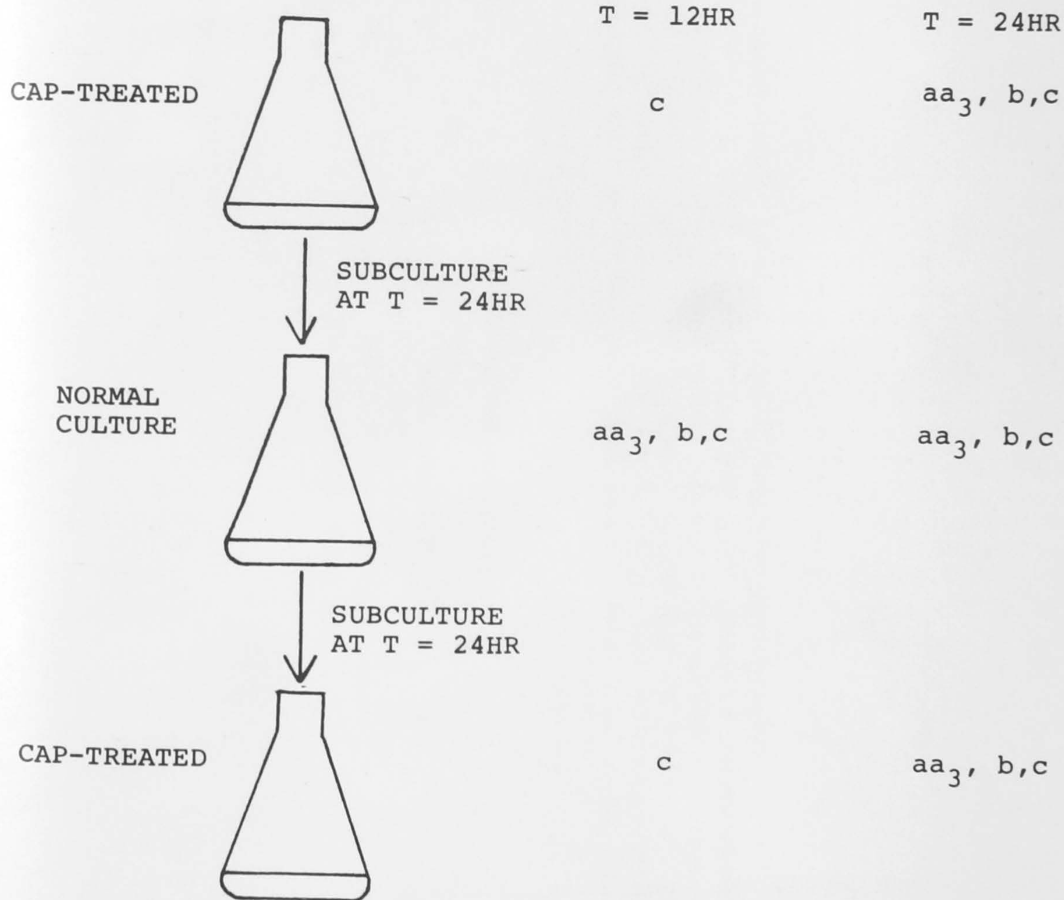
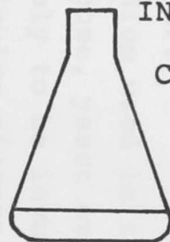

ABSOLUTE CYTOCHROME SPECTRA
OF CELLS AT
T = 12HR
T = 24HR
CAP-TREATED
c
aa3, b,c
SUBCULTURE
AT T = 24HR
NORMAL
CULTURE
aa3, b,c
aa3, b,c
SUBCULTURE
AT T = 24HR
CAP-TREATED
c
aa3, b,c

Figure 3-2. Nongenetic nature of the phasic phenomenon. Cells of a CAP-treated culture were subcultured at time T=12 and T=24 hr into fresh 2.5% glucose *Saccharomyces* media containing chloramphenicol (3 mg/ml) at an initial inoculum of 0.03 mg dry wt cells/ml culture. Subcultures were allowed to grow and cells of subcultures were examined for cytochrome contents at times T=12 and T=24 hr. For clarity of presentation, aa_3, b, c and c have the same meanings as explained in Figure 3-1.

ABSOLUTE CYTOCHROME SPECTRA OF CELLS AT

T = 12HR | T = 24HR

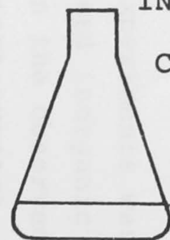
CAP-TREATED CULTURE



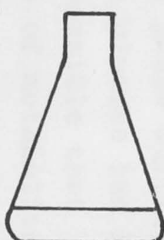
AT 12HR SUBCULTURE INTO FRESH MEDIUM CONTAINING CHLORAMPHENICOL

c | aa_3, b, c

CAP-TREATED CULTURE



AT 24HR SUBCULTURE INTO FRESH MEDIUM CONTAINING CHLORAMPHENICOL



c | aa_3, b, c

When the same experiments were carried out using erythromycin (ER) in place of CAP, an identical set of responses was obtained.

The importance of the culture environment in the apparent change of cellular sensitivity towards inhibition by CAP and ER on the synthesis of cytochromes aa_3 and b was suggested by the experimental result shown in Figure 3-3. Subculture of early logarithmic phase cells of a CAP-treated culture into fresh medium containing either CAP or ER for 5 or 6 hr resulted in the inhibition of synthesis of cytochromes aa_3 and b. However, subculture of the same cells into a spent medium (stationary phase environment) with added CAP or ER resulted in the resynthesis of cytochromes aa_3 and b.

Assay of glucose in the stationary phase culture medium used for the subculture indicated that glucose had been completely consumed. It thus seemed possible that exhaustion of glucose, or accumulation of metabolites from glucose might be the cause of the change in sensitivity of cytochrome synthesis to CAP and ER. This is unlikely, however, since replacement of glucose by glycerol, lactate or ethanol in the fresh culture medium did not change cellular sensitivity towards inhibition by CAP as shown by the inability to resynthesise cytochromes aa_3 and b after subculturing for 6 hr.

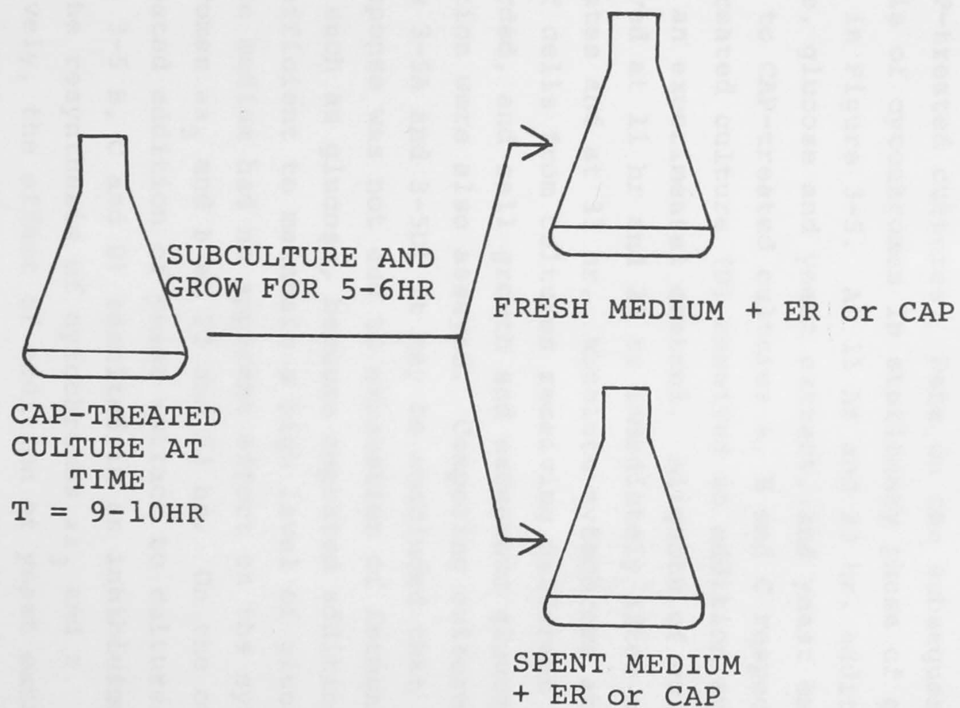
The culture medium used in these experiments was composed essentially of glucose, yeast extract and inorganic salts. The components likely to be involved in the observed phasic phenomenon in particular glucose and yeast extract, were individually tested.

3-3-2 EFFECT OF YEAST EXTRACT AND GLUCOSE ON THE PHASIC PHENOMENON

In an examination of the effect of initial concentration of yeast extract in the culture medium on the synthesis of cytochromes aa_3 and b at stationary phase of growth, additional yeast extract stimulated growth substantially in normal cultures. On the other hand, additional yeast extract consistently and signficantly inhibited growth in

Figure 3-3. Environmental control of the phasic phenomenon. Cells of a CAP-treated (3 mg/ml) culture of *C. parapsilosis* were subcultured into fresh 2.5% glucose *Saccharomyces* medium containing CAP (3 mg/ml) or ER (5 mg/ml) and into spent medium containing added CAP (3 mg/ml) or ER (5 mg/ml) at the same cell density as the original CAP-treated culture at time T=9-10 hr, that is at about 1 mg dry wt cells per ml. These subcultures were allowed to grow further for 5-6 hr and cytochrome contents of these cells were examined. Spent medium was the cell-free medium of a normal culture of *C. parapsilosis* grown in 2.5% glucose *Saccharomyces* medium to time T=12 hr. Effect of replacing glucose in the fresh medium by other carbon source on the synthesis of cytochromes in CAP-treated cultures is shown in the insert.

	CARBON SOURCE USED IN THE FRESH *SACCHAROMYCES* MEDIUM OF SUBCULTURE	ADDITION TO SUBCULTURE	CYTOCHROMES OBSERVED IN CELL SPECTRA
SUBCULTURE LOGARITHMIC-PHASE CELLS OF A CAP-TREATED CULTURE AND GROW FOR 6 HR ON SUB-CULTURE	→ Glucose, 2.5% (w/v)	Nil	aa_3, b, c
		+CAP (3mg/ml)	c
	→ Glycerol, 5.0% (w/v)	Nil	aa_3, b, c
		+CAP	c
	→ Lactate, 5.0% (w/v)	Nil	aa_3, b, c
		+CAP	c
	→ Ethanol, 3.0% (v/v)	Nil	aa_3, b, c
		+CAP	c



ABSOLUTE CYTOCHROME ABSORPTION
SPECTRA OF CELLS OF SUBCULTURES
AT TIME T = 15HR

c

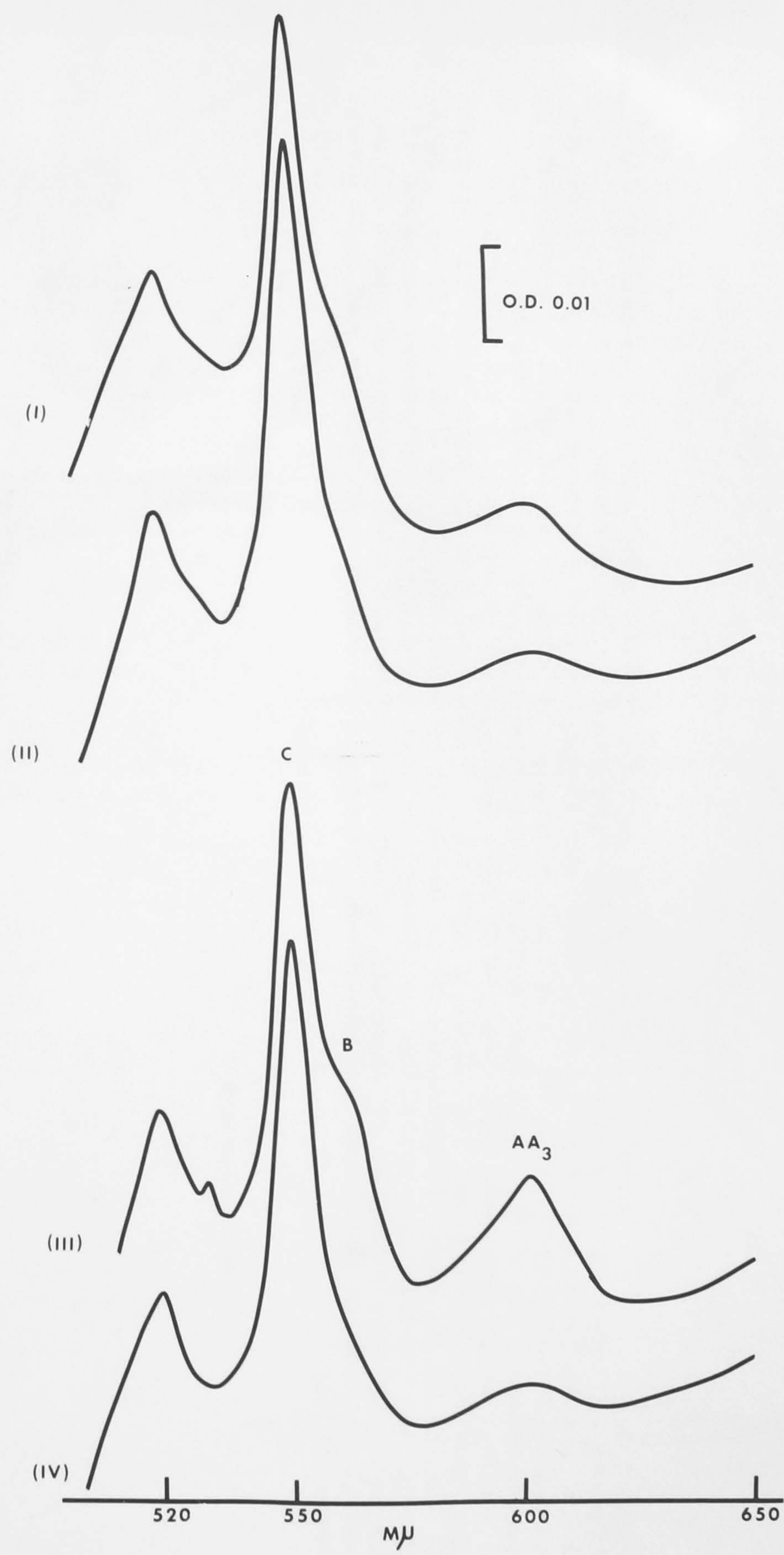
aa_3, b, c

CAP-treated cultures. When absolute cytochrome absorption spectra of cells cultured in the presence of CAP on high and normal concentrations of yeast extract were examined, it was found that additional yeast extract inhibited the resynthesis of cytochromes aa_3 and *b* in stationary phase cells (Figure 3-4). It appeared that the inhibition of cell growth by CAP on high concentrations of yeast extract may have been due to the restricted ability to resynthesise cytochromes aa_3 and *b*. It also suggested that changes of concentration of some components present in the yeast extract during growth may be responsible for the phasic response of cells towards inhibitors.

In order to test this possibility, further additions of glucose and yeast extract were made to logarithmic-phase, CAP-treated cultures. Data on the subsequent resynthesis of cytochromes in stationary phase of growth are shown in Figure 3-5. At 11 hr and 23 hr, additions of glucose, glucose and yeast extract, and yeast extract were made to CAP-treated cultures A, B and C respectively. One CAP-treated culture (D) received no addition and served as an experimental control. Aliquots of cultures were removed at 11 hr and 23 hr immediately after addition of substrates and at 33 hr. Absolute cytochrome absorption spectra of cells from cultures receiving different treatments were recorded, and cell growth and exogenous glucose concentration were also assayed. Comparing cultures A and D (Figures 3-5A and 3-5D) it may be concluded that the phasic response was not due to exhaustion of fermentable substrate such as glucose, because repeated addition of glucose sufficient to maintain a high level of glucose in the culture medium had no apparent effect on the synthesis of cytochromes aa_3 and *b* at 23 and 33 hr. On the other hand, repeated addition of yeast extract to cultures B and C (Figures 3-5 B, C and D) resulted in an inhibition or delay of the resynthesis of cytochromes aa_3 and *b*. Quantitatively, the effect of addition of yeast extract on the synthesis of cytochromes in stationary phase cells of a CAP-treated culture is shown in Table 3-1. Synthesis of cytochromes aa_3 and *b* but not cytochrome *c* was inhibited by the addition of yeast extract. It seemed therefore that

Figure 3-4. Effect of initial concentrations of yeast extract on the synthesis of cytochromes in CAP-treated cultures.
Cells of two CAP-treated cultures of *C. parapsilosis* grown in 0.5 and 2.5% (w/v) initial concentrations of yeast extract were examined for cytochrome contents at times T=12 and T=24 hr. Curves (I) and (II) are the cytochrome absorption spectra of cells of CAP-treated cells grown in 2.5% yeast extract at time T=24 and 12 hr respectively, and Curves (III) and (IV) are those grown in 0.5% yeast extract at time T=24 and T=12 hr respectively. Peaks denoted AA_3, B and C represent α-absorption bands of cytochromes aa_3, *b* and *c* respectively. The effect of initial concentrations of yeast extract on cell growth of CAP-treated cultures is shown in the insert.

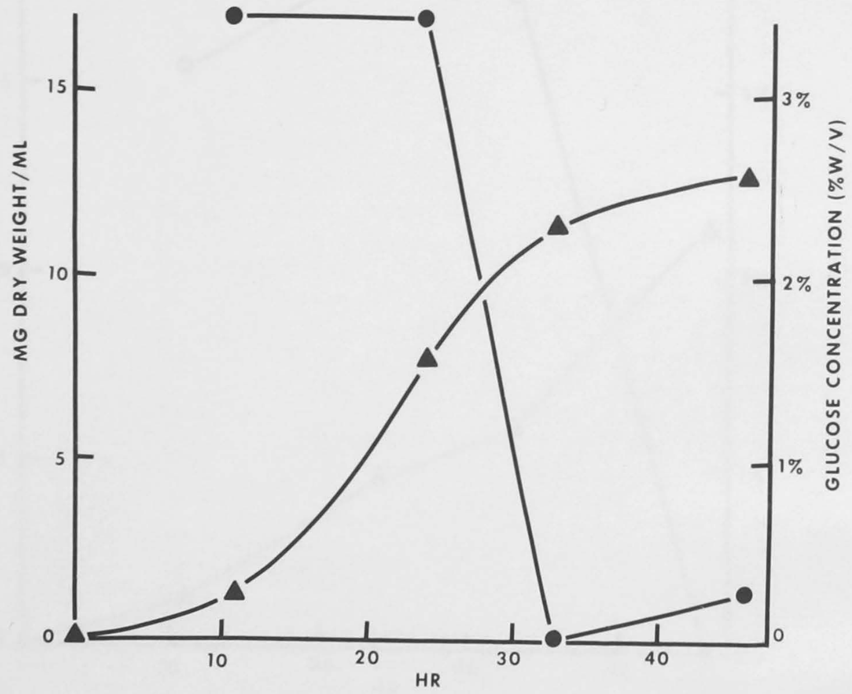
INITIAL CONCENTRATION OF YEAST EXTRACT IN CULTURE MEDIUM	CELL GROWTH (mg dry wt cells/ml) AT	
	T=12 HR	T=24 HR
Control		
0.5%	4.5	9.8
2.5%	4.8	15.0
CAP-treated		
0.5%	1.3	6.9
2.5%	1.8	5.0

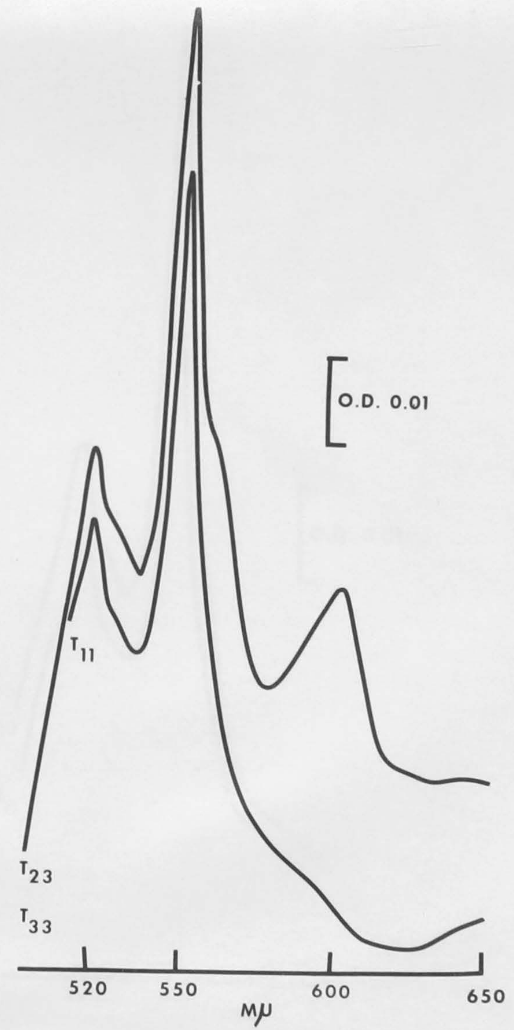


O.D. 0.01
(I)
(II)
C
B
AA3
(III)
(IV)
520
550
600
650
MμU

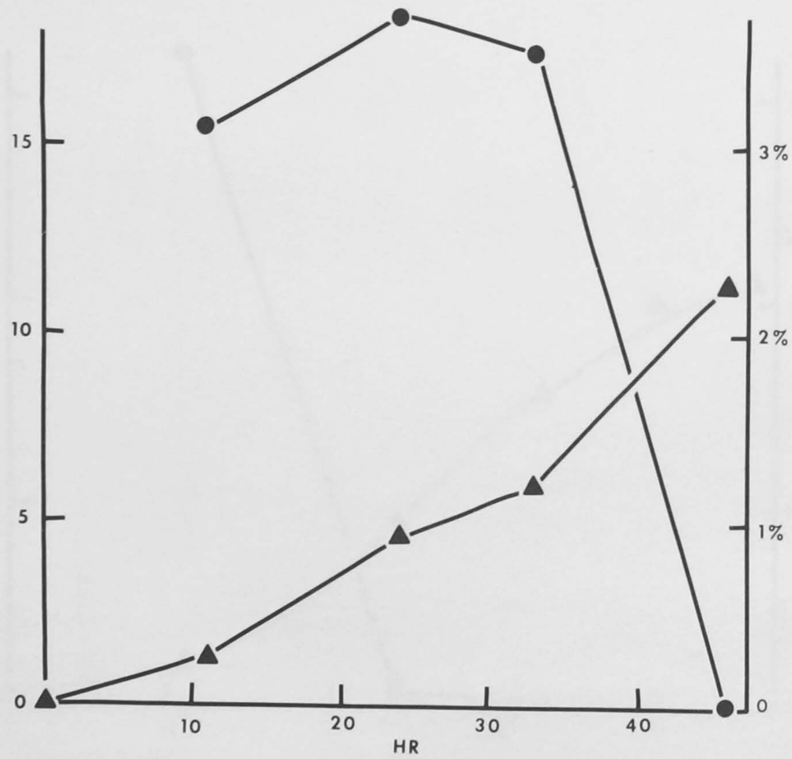
Figure 3-5. Effect of addition of glucose and/or yeast extract to growing CAP-treated cultures of *C. parapsilosis* on the synthesis of cytochromes.
A CAP-treated culture of *C. parapsilosis* was divided into 4 portions at time T=11 hr and these were designated as A, B, C and D. Additions of glucose (2.5% w/v), glucose and yeast extract (1% w/v), and yeast extract were made immediately to cultures A, B and C respectively; the same additions were made again after a further 12 hr. Culture D received no addition and served as a control. Cells were removed from these cultures at 11 hr and 23 hr (i.e. immediately after addition of substrates) and also at 33 hr. Cell growth and glucose concentration in the medium were determined. Cytochrome contents of these cells were examined by absolute cytochrome absorption spectroscopy of whole cells. Curves denoted by ●——● and ▲——▲ represent glucose concentration and cell growth respectively.

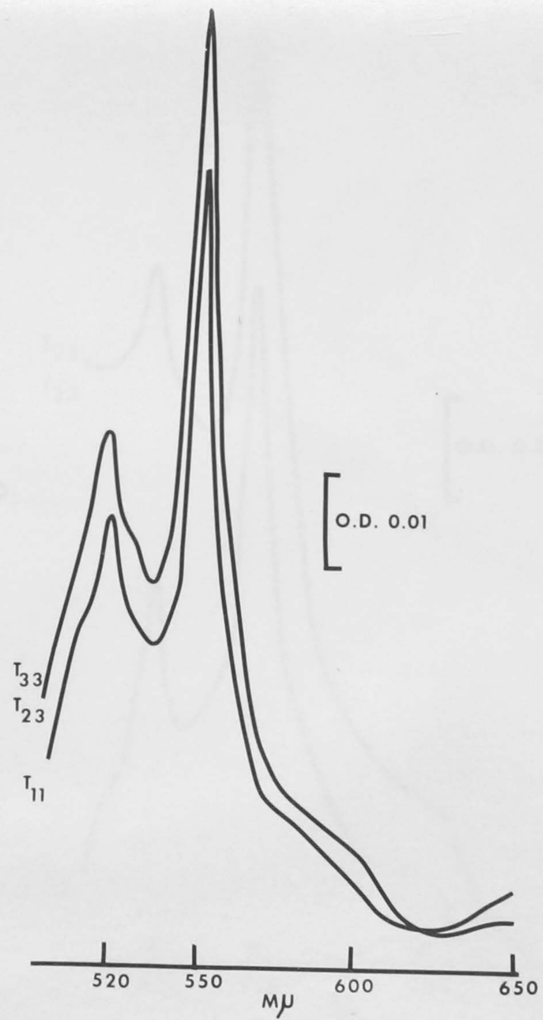
(A)



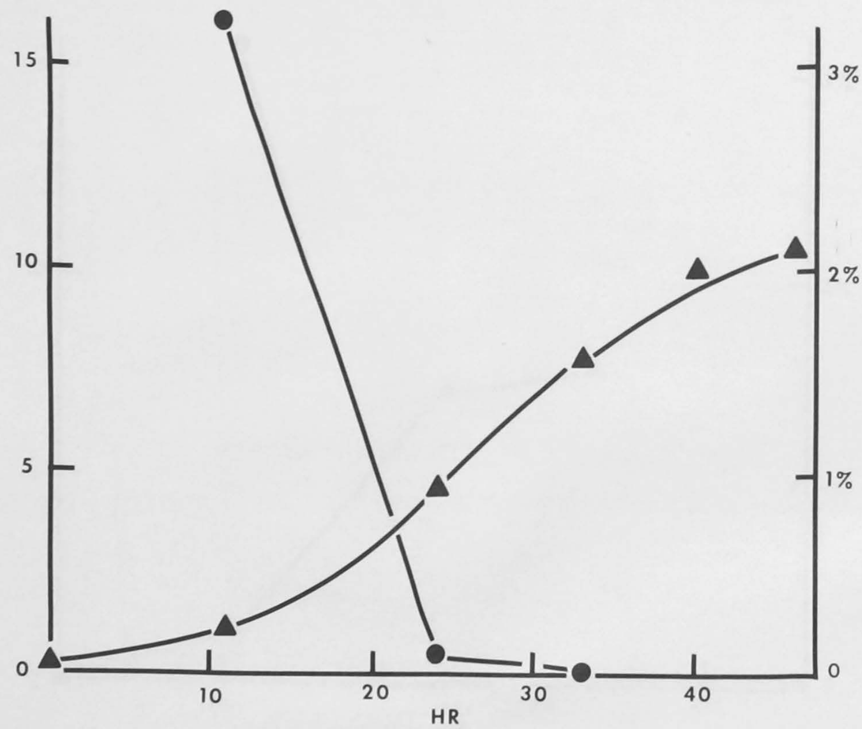


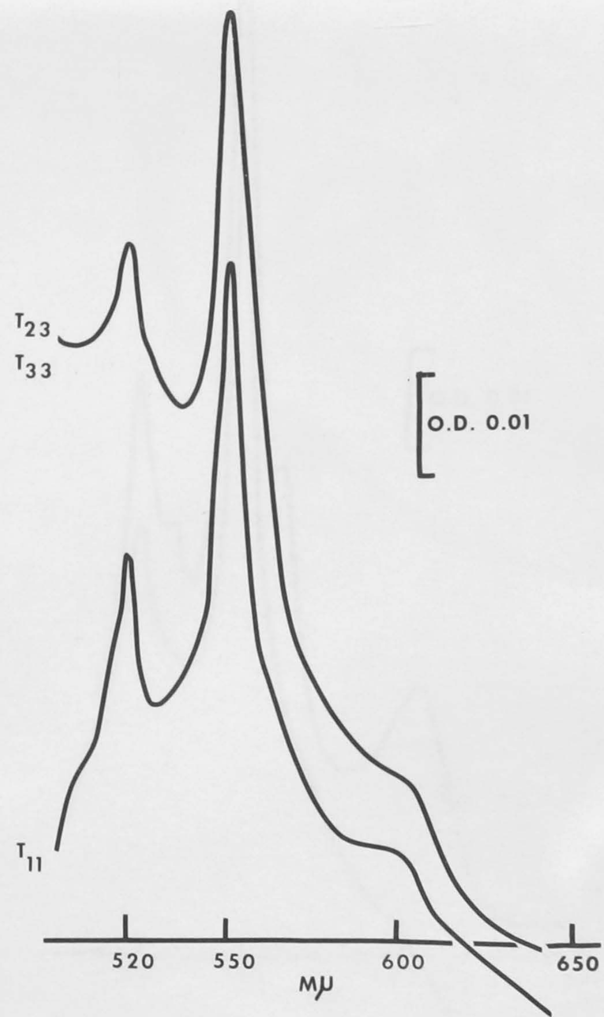
(B)





(C)





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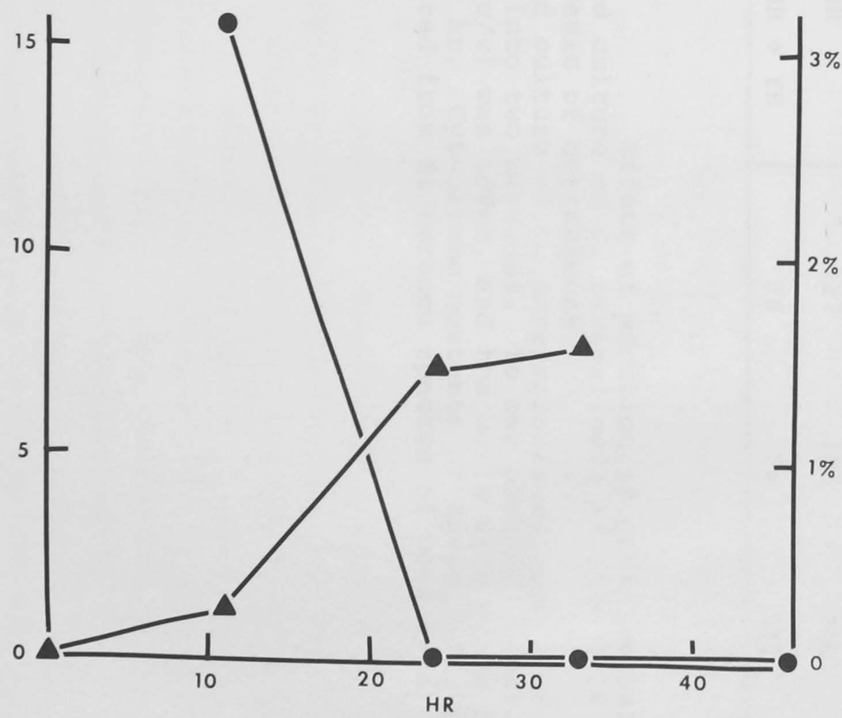
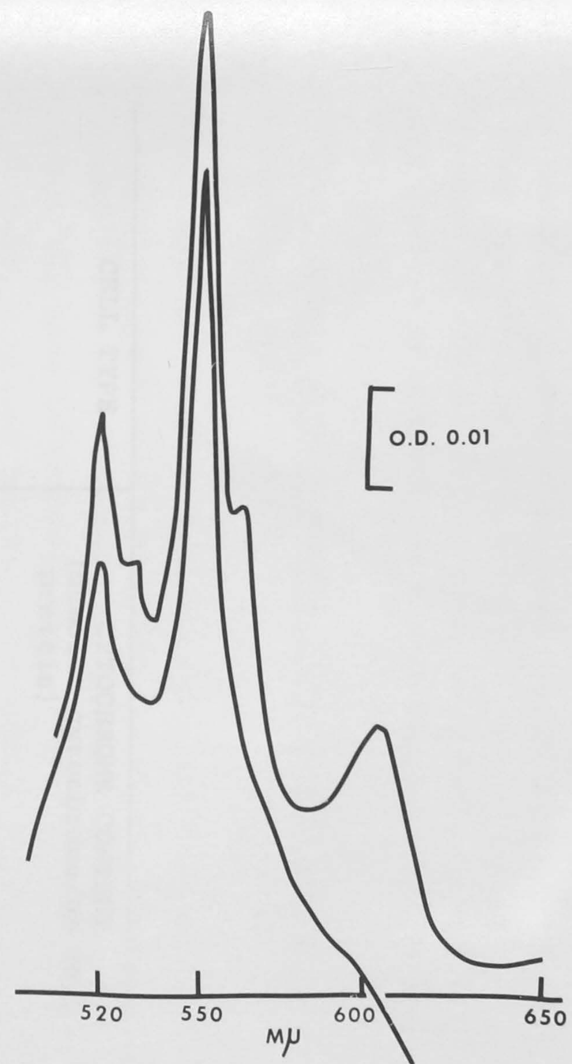




CELL TYPE	CYTOCHROME CONTENT (mμmoles cytochrome/gm cell protein)		
	aa_3	b	c
T=14 HR	40	90	382
T=24 HR	127	195	368
T=24 HR + YE	38	92	351

Table 3-1. Effect of addition of yeast extract to a CAP-treated culture of *C. parapsilosis* at time T=14 hr on the synthesis of cytochromes.
A CAP-treated culture of *C. parapsilosis* at time T=14 hr was divided into two portions. To one portion, yeast extract (1% w/v) was added, and the cells were grown further to time T=24 hr. Cytochrome contents of these cell types were calculated from difference spectra of cell-free homogenates.

the phasic phenomenon (as manifested by the apparent change in the sensitivity of cells towards inhibition by CAP and ER of the synthesis of cytochromes aa_3 and b) was a consequence of changes in the culture environment due to certain changes of activity or nutritional value of yeast extract.

3-3-3 RELATIONSHIP BETWEEN GROWTH RATE AND THE PHASIC PHENOMENON

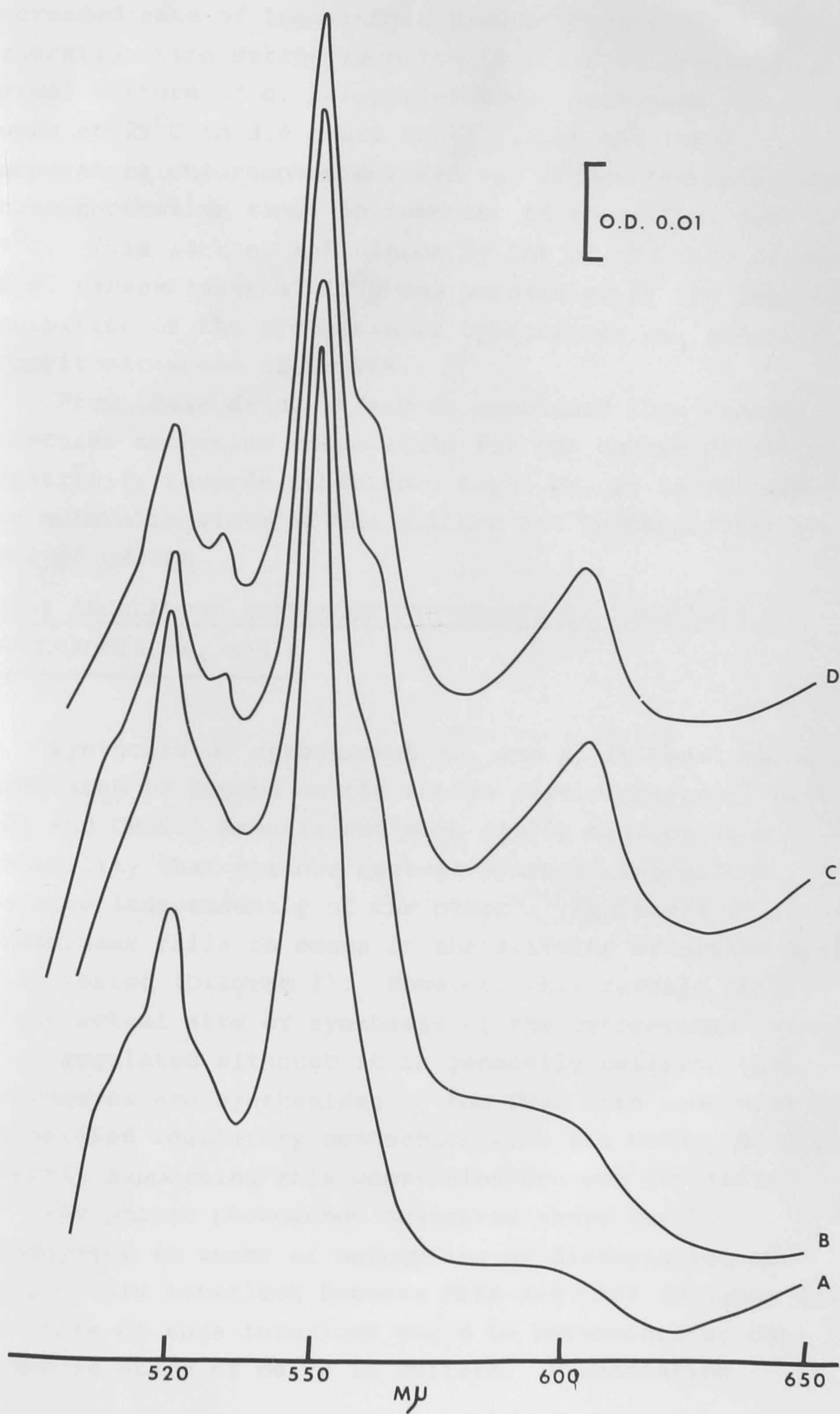
Yeast extract is a water-soluble extract of autolyzed yeast. Its use as a source of essential growth factors for yeasts and other microorganisms is well established. An attempt was made to identify the factors contained in yeast extract, critical for the growth of *C. parapsilosis* (Table 3-2). The organism was found to be auxotrophic for biotin; pantothenate gave considerable stimulation of growth without being essential. Addition of vitamins in excess quantity did not increase further cell growth, and could not mimic the growth promoting ability of yeast extract. It may be inferred that other growth-accelerating substances are present in the yeast extract besides B-vitamins. The possibility that certain essential growth factors (B-vitamins) might mimic the effect of yeast extract on the resynthesis of cytochromes aa_3 and b in stationary phase cells of CAP-treated cultures was examined. Results are shown in Figure 3-6. As before, addition of yeast extract to a CAP-treated culture at logarithmic-phase of growth (14 hr) inhibited cell growth and synthesis of cytochromes aa_3 and b at stationary phase. On the other hand addition of excess amounts of vitamins had no apparent effect on either cell growth or synthesis of cytochromes aa_3 and b at stationary phase of growth. From these results, it appeared that the conferring of drug-sensitivity towards CAP or ER in stationary-phase cells was related to the potentiality for accelerated or rapid cell division. In other words, the determinant of sensitivity to CAP was related in some way to the rate of cell growth. The effect of CAP on the synthesis of cytochromes was therefore examined in cultures in which the logarithmic rate of growth

CULTURE MEDIUM	CELL GROWTH AFTER 12 HR (mg dry wt cells/ml culture)
G,S	0.48
G,S,V	2.30
G,S,3V	2.54
G,S,3V minus calcium pantothenate	1.83
G,S,3V minus thiamine	2.12
G,S,3V minus inositol	2.20
G,S,3V minus pyridoxine hydrochloride	2.28
G,S,3V minus nicotinic acid	2.36
G,S,3V minus biotin	0.48
G,S,YE (i.e. 2.5% glucose *Saccharomyces* medium	6.75

Figure 3-2. Determination of the essential growth factors for *C. parapsilosis*.
2.5% glucose *Saccharomyces* medium was composed of glucose (G) at 2.5% W/V, mineral salts (S) at concentrations specified in Section 2-2-2, and yeast (YE) at 0.5% (w/v. Culture medium containing G and S was thus 2.5% glucose *Saccharomyces* medium with the omission of yeast extract. Culture media containing G,S, and V had additional vitamin cocktail consisting of calcium pantothenate, thiamine, inositol, pyridoxine hydrochloride, nicotinic acid and biotin in concentrations specified in Section 3-2-1; and G,S,3V had vitamin supplements at three times the concentrations specified in Section 3-2-1. In testing vitamins for their growth supporting ability, individual vitamins were in turn omitted from the culture medium designated G,S,3V; for example, culture medium designated "G,S,3V minus calcium pantothenate" did not contain calcium pantothenate. Standard inoculum for these cultures were 0.03 mg/ml, and cell yields at 12 hr, representing the end of logarithmic phase of growth, were taken for comparison.

Figure 3-6. Failure of B-vitamins to mimic the effect of the addition of yeast extract to a CAP-treated culture on the synthesis of cytochromes aa_3 and *b*. A CAP-treated culture of *C. parapsilosis* was divided into 3 portions at time T=14 hr. Additions of yeast extract (1% w/v) and B-vitamins (5 times the concentrations specified in Section 3-2-1) were made to two individual portions, and one portion received no addition. These cultures were further grown to time T=23 hr and cytochrome contents of these cells were examined by absolute cytochrome absorption spectroscopy of whole cells. Growth of these cultures was also determined, and is shown in the insert. Curves A, B, C and D are cytochrome absorption spectra of CAP-treated cells at T=14 hr, and CAP-treated cells at T=23 hr receiving addition of yeast extract, addition of vitamins, and no addition respectively.

CULTURE	CELL GROWTH (mg dry wt cells/ml)
At T=14 HR	
2.5% glucose *Saccharomyces* medium plus CAP (3 mg/ml)	1.93
At T=23 HR	
2.5% glucose *Saccharomyces* medium plus CAP	6.75
2.5% glucose *Saccharomyces* medium plus CAP and 1% YE	3.08
2.5% glucose *Saccharomyces* medium plus CAP and V(5x)	5.80
At T=25 HR	
2.5% glucose *Saccharomyces* medium plus CAP	6.70
2.5% glucose *Saccharomyces* medium plus CAP and 1% YE	3.52
2.5% glucose *Saccharomyces* medium plus CAP and V(5x)	6.57


O.D. 0.01
D
C
B
A
520
550
600
650
mμ

was reduced. Reduced temperature was used to effect a decreased rate of logarithmic growth (Figure 3-7). The generation time during logarithmic phase of growth of a normal culture of *C. parapsilosis* was prolonged from 1.25 hours at 29°C to 3.4 hours at 18°C. At the lower temperature chloramphenicol did not affect the logarithmic-phase generation time, in contrast to the effect seen at 29°C. This lack of inhibition by CAP of the rate of growth of *C. parapsilosis* at 18°C was paralleled by the lack of inhibition of the synthesis of cytochromes aa_3 and *b* at logarithmic-phase of growth.

From these data, it may be concluded that whatever the molecular mechanism responsible for the change of cellular sensitivity towards inhibitors might be, it is related to the metabolic state of the culture and in particular to the rate of growth.

3-3-4 REGULATORY INTERLOCK HYPOTHESIS OF SYNTHESIS OF CYTOCHROMES aa_3 and *b*

Synthesis of cytochromes aa_3 and *b* in yeast has been postulated to depend on the active participation of both MPSS and CPSS. Ashwell and Work (1970) mention "the probability that neither protein-synthesising system operates independently of the other". Synthesis of cytochromes fails to occur if the activity of either system is inhibited (Diagram 1). However, this reveals nothing of the actual site of synthesis of the cytochromes nor how it is regulated although it is generally believed that cytochromes are synthesized by the CPSS with some sort of unspecified regulatory connection with the MPSS. No data directly supporting this conviction are yet available.

The phasic phenomenon described above may be interpreted in terms of uncoupling or dissociation of a regulatory interlock between MPSS and CPSS (Diagram 2). The state of this interlock could be determined by the metabolic state of cells in culture. Dissociation

Figure 3-7. Effect of reduction of growth rate by lowering temperature of cultivation on the inhibition of synthesis of cytochromes *aa*$_3$ and *b* by CAP. *C. parapsilosis* was cultivated in *Saccharomyces* media containing 2.5% (w/v) glucose and 2.5% (w/v) glucose plus CAP (3 mg/ml) at 18°C. Effect of CAP on the logarithmic phase generation time (see insert) and synthesis of cytochromes was determined.

CULTURE TYPE	LOGARITHMIC PHASE GENERATION TIME (HR)	CELL GROWTH AT (mg dry wt cells/ml)	
		T=20 HR	T=24 HR
2.5% glucose *Saccharomyces* medium	3.4	0.37	0.93
2.5% glucose *Saccharomyces* medium plus CAP	3.4	0.35	0.76

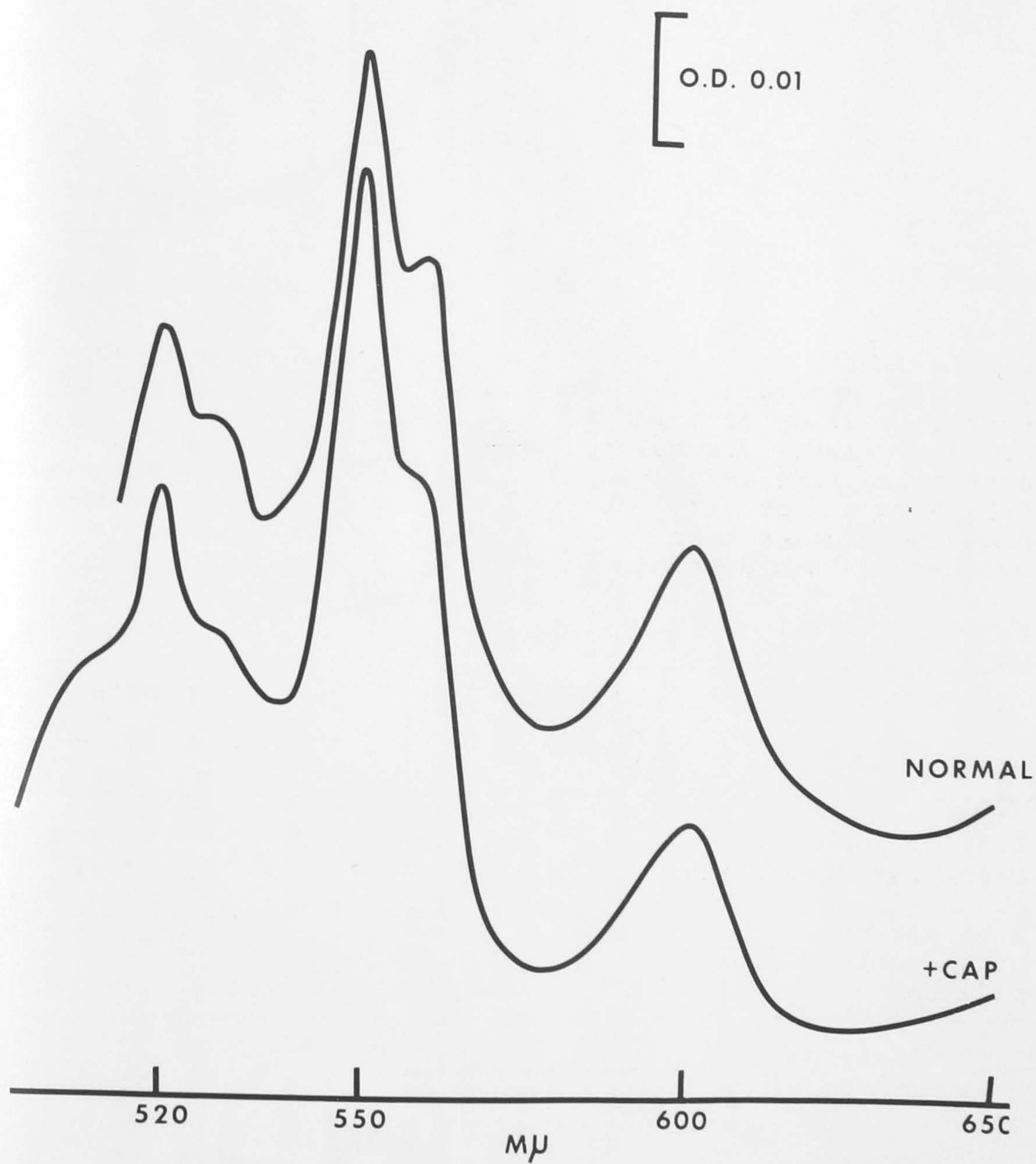
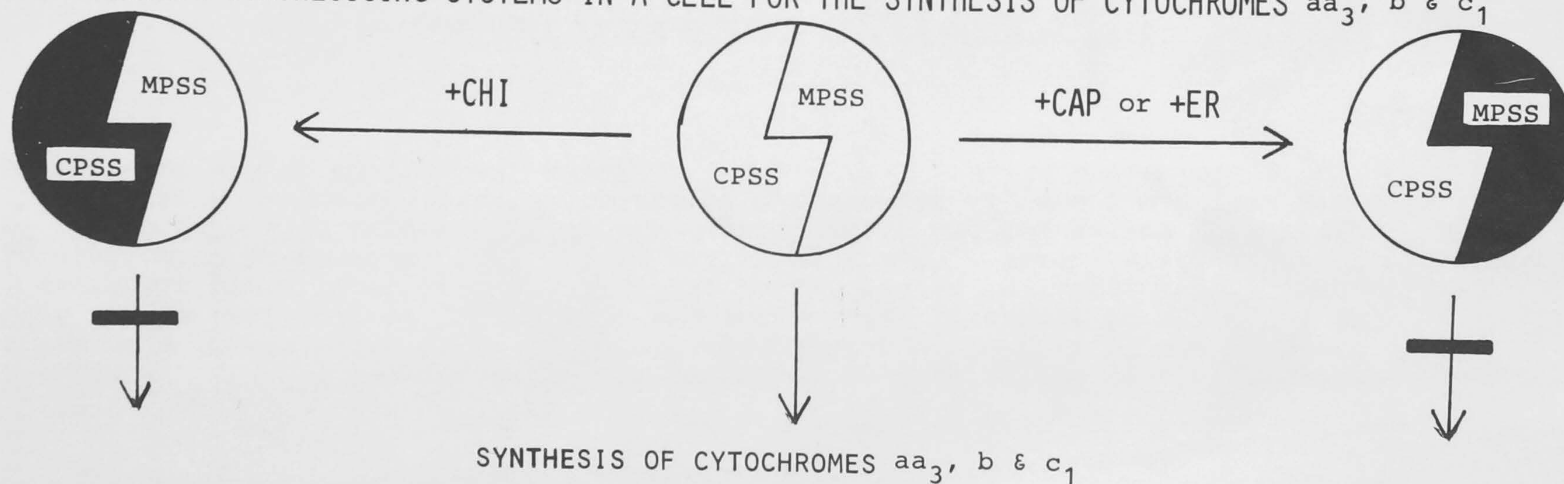

O.D. 0.01
NORMAL
+CAP
520
550
600
650
Mμ

Diagram 1. Synthesis of cytochromes aa_3, b and c_1 in a cell has been said to require active participations of the MPSS and CPSS.
Inhibition of the CPSS by CHI or of the MPSS by CAP (or ER) results in the inhibition of the synthesis of cytochromes aa_3, b and c_1. Hence neither systems can act independently of the other in the synthesis of the cytochromes. An interlock has been envisaged. The interlock associating activities of the mitochondrial and cytoplasmic protein synthesising systems is represented by the interdigitation of the two halves of a circle.

DIAGRAMATIC REPRESENTATION OF AN INTERLOCK ASSOCIATING THE ACTIVITIES OF THE TWO PROTEIN-SYNTHESISING SYSTEMS IN A CELL FOR THE SYNTHESIS OF CYTOCHROMES aa_3, b & c_1



MPSS = MITOCHONDRIAL PROTEIN SYNTHESISING SYSTEM

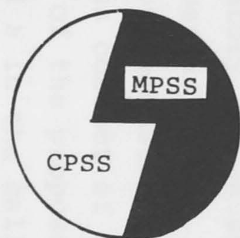
CPSS = CYTOPLASMIC PROTEIN SYNTHESISING SYSTEM

SHADING REPRESENTS INHIBITION

Diagram 2. Experimental data described in this thesis can be explained in terms of a dissociation of an interlock associating activities of the MPSS and CPSS in the synthesis of cytochromes *aa*$_3$ and *b*. The interlock is effective in logarithmic phase (i.e. actively dividing cells) but it is ineffective or dissociated in stationary phase (i.e. nondividing cells) of growth. Dissociation or ineffectiveness of the interlock is represented by the separation of the two halves of a circle. When the interlock is ineffective, synthesis of cytochromes *aa*$_3$ and *b* can take place in the presence of CAP or ER.

SCHEMATIC INTERPRETATION OF EXPERIMENTAL DATA

LOGARITHMIC-PHASE



STATIONARY-PHASE

SYNTHESIS OF CYTOCHROMES aa_3 & b

may be induced when the rate of cell-division declines or when the potentiality for rapid cell division is low. Association of the interlock could be reinstated by increasing the rate of cell division.

It is not difficult to propose experimental tests of my hypothesis. A few corollaries of the hypothesis which could be experimentally verified are:-

(I) It is necessary that there be no change in cellular permeability to CAP or ER; there should be no gross inactivation or destruction of the antibiotic; and there should be no particular change in the sensitivity of the mitochondrial protein synthesis towards CAP or ER.

(II) Since the proposed hypothesis specifies that the site of synthesis of cytochromes aa_3 and b is the CPSS and in the stationary phase of growth the interlock becomes dissociated, synthesis of the cytochromes in stationary phase cells should not therefore be affected by any further additions of inhibitors of the mitochondrial protein synthesis to a CAP- or ER-treated culture. This synthesis should be inhibited by inhibitors of cytoplasmic protein synthesis.

(III) If the phasic phenomenon is intrinsically an expression of a regulatory mechanism operating on the synthesis of cytochromes aa_3 and b, involving the MPSS and CPSS, then it should be demonstrable in normal cultures, independent of previous contact of cells with CAP or ER.

These corollaries of the proposed hypothesis of synthesis of cytochromes aa_3 and b in the cell were tested as described in following sections.

3-3-5 PERMEABILITY OF CELLS TO CHLORAMPHENICOL

The permeability of cells towards chloramphenicol in logarithmic and stationary phase of growth of the normal and CAP-treated cultures was tested by measuring uptake of labelled chloramphenicol. The rate of uptake was found to be very rapid for both control and CAP-treated cells, near-maximal uptake being reached after incubation for 1 min, the shortest time tested. Uptake of chloramphenicol was measured by the amount of radio-activity bound to cells after

6 washes with ice-cold water. Control experiments showed that a constant level of bound radio-activity was reached after washing incubated cells 5-6 times with cold water at $0^{o}C$. 80% of this cold-wash residual uptake could be removed by washing with warm medium or water at $35^{o}C$; the remainder appeared to be irreversibly bound.

Table 3-3 shows the uptake of CAP by cells when the external CAP concentration in the incubation medium was very low (0.063 mg/ml). It was felt that if there was any difference in permeability between logarithmic and stationary phase cells of both normal and CAP-treated cultures, they should show marked differences at low external CAP concentrations. As is apparent from Table 3-3, there was no significant difference in uptake of CAP between untreated cells from either logarithmic or stationary phase of growth, and the stationary phase cells of both the control and CAP-treated cultures showed significantly higher uptakes of CAP than the corresponding logarithmic phase cells. Furthermore, when uptake of CAP was measured over a wide range of external CAP concentrations (up to 4 mg/ml) these general conclusions were still found to hold (Figure 3-8).

3-3-6 ASSAY OF CHLORAMPHENICOL IN CULTURE MEDIUM

Direct assay of unconjugated or active form of CAP in in culture medium by two independent methods showed (Levine and Fischbach, 1951; Hughes and Diamond, 1964) that (Table 3-4) there was no substantial change in amount of the active form of CAP in the medium from logarithmic and stationary phase culture.

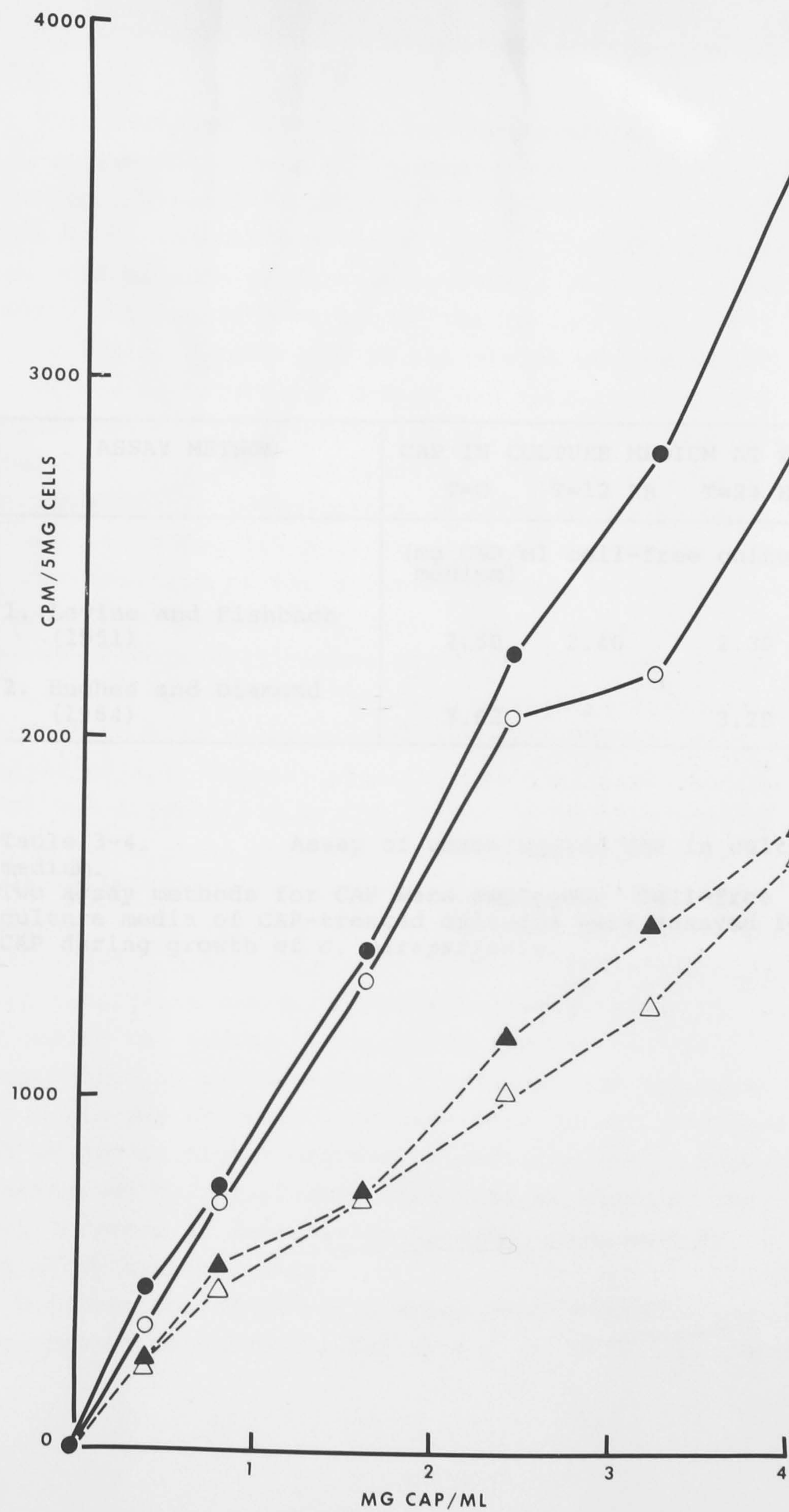
3-3-7 *IN VITRO* AND *IN VIVO* ASSESSMENT OF MITOCHONDRIAL PROTEIN SYNTHESIS

The question of whether or not the mitochondrial protein synthesising system *in vivo* alters in its sensitivity towards chloramphenicol and erythromycin during the culture cycle was examined by measurement of the activity of the system both *in vitro* (Lamb *et al.*, 1968) and *in vivo* (Schatz and Saltzgaber, 1969).

CELL TYPE	CAP UPTAKE BY CELLS (cpm/5 mg dry wt cells after incubation for 1 hr)
NORMAL	
T=12 HR	1000
T=24 HR	1500
CAP-TREATED	
T=12 HR	850
T=24 HR	1400

Table 3-3. Uptake of C^{14}-CAP by cells at low external CAP concentration (0.063 mg/ml; 2μCi/0.063 mg CAP).
Cells were removed from normal and CAP-treated cultures at times T=12 and T=24 hr, washed with warm water and then incubated with C^{14}-CAP at 30°C. Cells, after incubation, were washed with ice-cold water and processed for determination of radio-activities.

Figure 3-8. Uptake of CAP by logarithmic and stationary phase cells of a normal and CAP-treated cultures of *C. parapsilosis*.
Cells were removed from normal and CAP-treated (3 mg/ml) cultures of *C. parapsilosis* at time T=11 and T=24 hr, and washed. Washed cells were incubated for 1 hr at $30^{o}C$ in *Saccharomyces* media containing 2.5% glucose and C^{14}-CAP of constant specific radio-activity (1 μCi/3mg CAP) at different concentrations of CAP. After incubation, cells were immediately cooled and processed for determination of radio-activity. Curves ●——●, ○——○, ▲----▲ and △----△ represent uptake of CAP by normal and CAP-treated cells at time T=24 hr and time T=11 hr respectively. ●——●, normal at T=24 hr; ○——○, CAP-treated at T=24 hr; ▲----▲, normal at T=11 hr; and △----△, CAP-treated at T=11 hr.



4000
3000
2000
1000
0
CPM/5MG CELLS
1
2
3
4
MG CAP/ML

ASSAY METHOD	CAP IN CULTURE MEDIUM AT TIMES		
	T=O	T=12 HR	T=24 HR
	(mg CAP/ml cell-free culture medium)		
1. Levine and Fishbach (1951)	2.50	2.40	2.30
2. Hughes and Diamond (1964)	3.63	-	3.29

Table 3-4. Assay of unconjugated CAP in culture medium.
Two assay methods for CAP were employed. Cell-free culture media of CAP-treated cultures were assayed for CAP during growth of *C. parapsilosis*.

(i) *In vitro*

When isolated mitochondria were incubated with a radio-active amino acid (C^{14}-leucine) under appropriate conditions (Table 3-5) radio-activity was incorporated into hot TCA-insoluble material. This incorporation was taken as a measure of protein synthesis *in vitro*. This activity was found to be linear for up to 20 minutes (Figure 3-9). Furthermore if the number of counts per unit volume of incubation medium was kept constant the absolute amounts of radio-active amino acid incorporated varied inversely with its specific activity (Figure 3-10). This can probably be explained in terms of an effect of mass action, since for a given input of radio-activity, the concentration of the amino acid would be the higher the lower its specific activity. Characteristics of an *in vitro* mitochondrial amino acid incorporation system are shown in Table 3-5. The incorporation was completely dependent upon an external supply of ATP, independent of added pH 5.1 enzymes, highly sensitive to inhibition by CAP but insensitive to inhibition by cycloheximide.

In vitro amino acid incorporating activities of mitochondrial fractions isolated from logarithmic and stationary phase cells of a CAP-treated culture are shown in Table 3-5. Apart from minor differences in specific activities, there was no significant modification of the MPSS during the growth cycle of CAP-treated culture. Incorporation by mitochondrial fractions from stationary phase cells was slightly less sensitive to CAP, but this might be due to higher degrees of mitochondrial damage and contamination by cytoplasmic ribosomes as shown by the slight increase in sensitivity towards inhibition by RNase or by cycloheximide.

A comparison of *in vitro* amino acid incorporating activities of mitochondria isolated from stationary phase

Figure 3-9. Kinetics of *in vitro* incorporation of C^{14}-leucine by mitochondria.
Mitochondria were prepared by lysis of protoplasts of logarithmic phase *C. parapsilosis* obtained by digestion of the cell wall with snail-gut enzymes. Mitochondria were obtained by lysis of the protoplasts and differential centrifugation of the resultant lyzate. Incorporation of C^{14}-leucine was carried out in the complete system containing incubation buffer (KCl 0.2M, $MgCl_2$ 0.026M, TrisHCl 0.07M pH 7.4) 0.5 ml, ATP-regenerating system (ATP 30 mg, PEP 50 mg, pyruvate kinase 0.04 ml, and oligomycin 0.5 mg in 3 ml of solution) 0.1 ml, mitochondrial suspension (approximately 10 mg protein/ml) 0.1 ml, L-leucine-2-C^{14} (10 µCi/ml, 156 µCi/µmole) 0.02 ml and 0.2 ml of H_2O at 30°C for the required times. Incorporation was terminated by adding 5 ml of ice-cold 7% TCA containing D,L-leucine and left overnight. TCA-precipitates were processed for counting. Efficiency of counting was routinely over 95% for C^{14} isotopes.

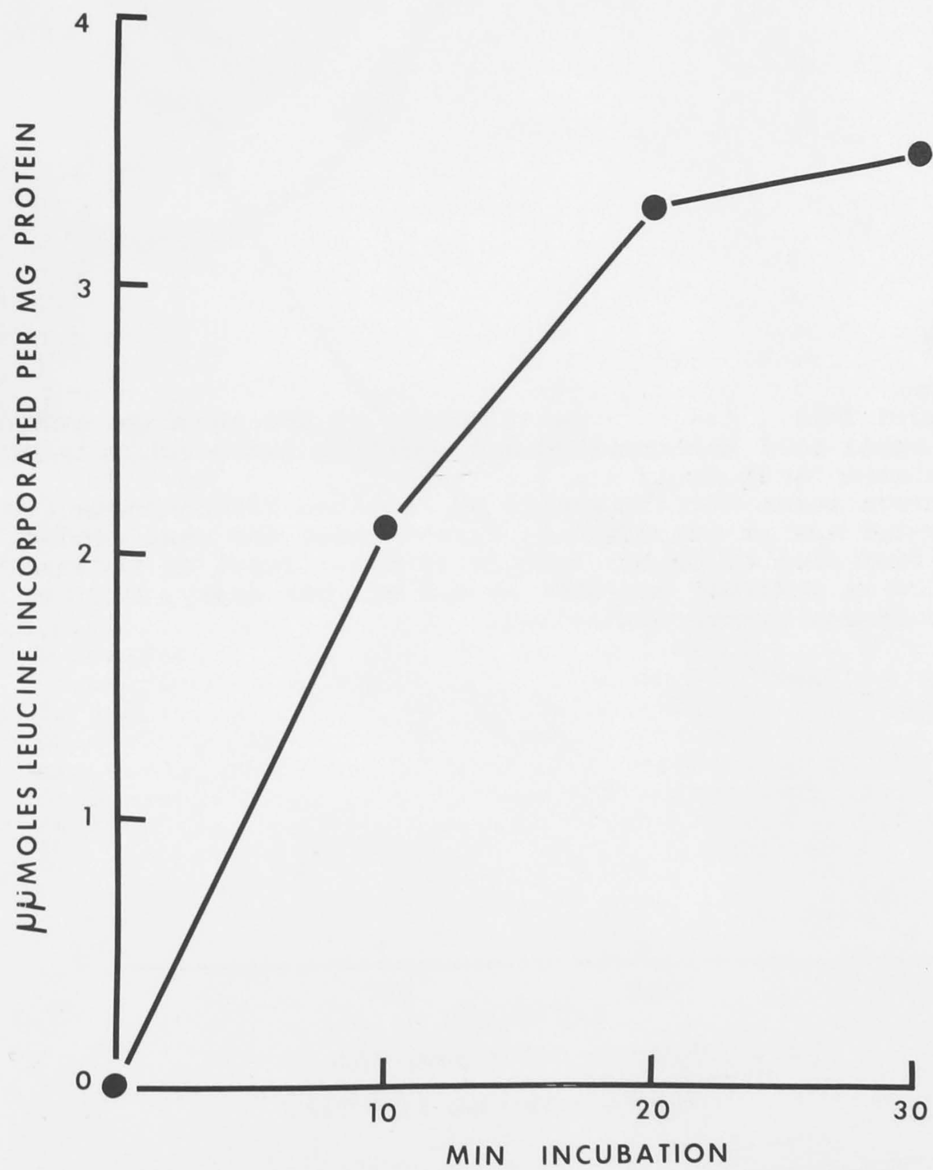
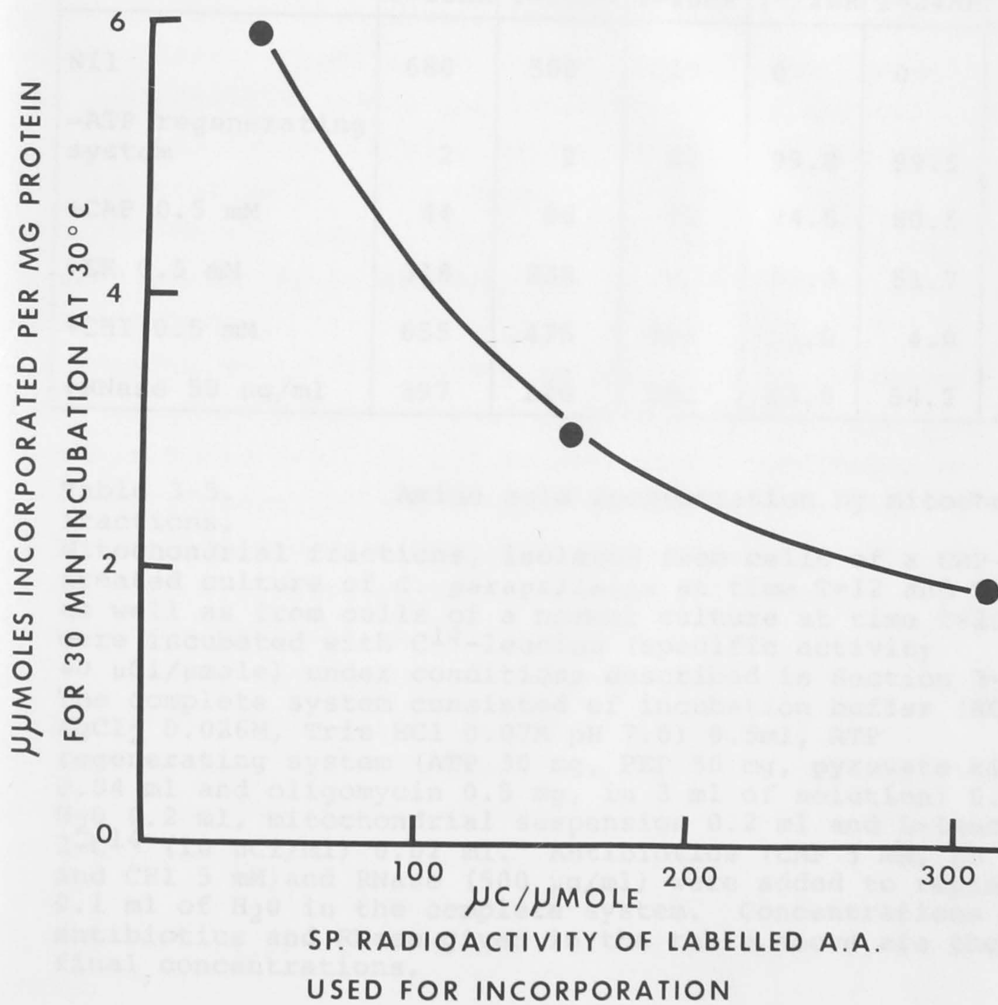

μμMOLES LEUCINE INCORPORATED PER MG PROTEIN
4
3
2
1
0
10
20
30
MIN INCUBATION

Figure 3-10. Relationship of the absolute amount of amino acid incorporated and specific radio-activity of the amino acid used.
Incorporation of C^{14}-leucine by isolated mitochondria was carried out in the complete system under the same conditions as described in Figure 3-9 for 30 min. Input of radio-activity was kept constant at 0.2 μCi per assay, but the specific activity was varied.



μμMOLES INCORPORATED PER MG PROTEIN
FOR 30 MIN INCUBATION AT 30°C
6
4
2
0
100
200
300
μC/μMOLE
SP. RADIOACTIVITY OF LABELLED A.A.
USED FOR INCORPORATION

ADDITION TO OR OMISSION FROM THE COMPLETE SYSTEM	C^{14} CPM INCORPORATED/ MG PROTEIN/30 MIN			% INHIBITION		
	CAP-TREATED AT		NORMAL AT	CAP-TREATED AT		NORMAL AT
	T=12HR	T=24HR	T=20HR	T=12HR	T=24HR	T=20HR
Nil	680	500	310	0	0	0
-ATP regenerating system	2	2	11	99.8	99.5	97
+CAP 0.5 mM	44	96	72	94.5	80.5	76
+ER 0.5 mM	318	238	-	53.0	51.7	-
+CHI 0.5 mM	655	475	304	3.0	4.0	0
+RNase 50 µg/ml	397	226	180	43.0	54.2	40

Table 3-5. Amino acid incorporation by mitochondrial fractions.
Mitochondrial fractions, isolated from cells of a CAP-treated culture of *C. parapsilosis* at time T=12 and T=24 hr as well as from cells of a normal culture at time T=20 hr, were incubated with C^{14}-leucine (specific activity 40 µCi/µmole) under conditions described in Section 3-2-3. The complete system consisted of incubation buffer (KCl 0.2M, $MgCl_2$ 0.026M, Tris HCl 0.07M pH 7.0) 0.5ml, ATP regenerating system (ATP 30 mg, PEP 50 mg, pyruvate kinase 0.04 ml and oligomycin 0.5 mg, in 3 ml of solution) 0.1 ml, H_2O 0.2 ml, mitochondrial suspension 0.2 ml and L-leucine-2-C^{14} (10 µCi/ml) 0.02 ml. Antibiotics (CAP 5 mM, ER 5 mM and CHI 5 mM)and RNase (500 µg/ml) were added to replace 0.1 ml of H_2O in the complete system. Concentrations of antibiotics and RNase given in the table above are therefore final concentrations.

cells of normal control and CAP-treated cultures (Table 3-5) supports the view that there was no significant modification of the sensitivity of the MPSS towards CAP in stationary phase cells.

(ii) *In vivo*

When a cell is pulsed with a radio-active amino acid, both CPSS and MPSS will incorporate the radio-active amino acid into their protein products. In the presence of a specific inhibitor of the CPSS, such as cycloheximide, at a concentration high enough to ensure complete inhibition of cytoplasmic protein synthesis, the MPSS remains active. The cycloheximide-resistant incorporation by the cell can thus be attributed largely to the activity of the MPSS. To characterise it fully however, it is necessary to demonstrate the sensitivity of this residual activity to inhibition by CAP or ER, and that it is localised in mitochondria.

Results of *in vivo* assays of the MPSS in logarithmic and stationary phase cells of a CAP-treated culture are shown in Table 3-6. It can be seen that logarithmic and stationary phase cells of a CAP-treated culture had cycloheximide resistant protein synthesising activities which were concentrated in mitochondrial fractions. In logarithmic and stationary phase cells these activities were strongly inhibited by erythromycin. This indicates that a MPSS with similar sensitivities towards ER was present in cells of both the logarithmic and the stationary phase of the CAP-treated culture. Also as shown in Table 3-6 the proportions of cycloheximide-resistant protein synthesis in cells of both phases of the CAP-treated culture were apparently higher (by 5-fold) than in control cells.

3-3-8 THE EFFECT OF INHIBITION OF PROTEIN SYNTHESIS BY ER AND CHI ON THE RESYNTHESIS OF CYTOCHROMES aa_3, b and c IN A CAP-TREATED CULTURE

A CAP-treated culture of *Candida* was grown for 16 hr. The culture was then divided into equal portions, and ER,

CELL TYPE	INHIBITOR ADDED	^{3}H-LEUCINE INCORPORATED (cpm/mg protein)		
		WH	MT	PMS
CAP-TREATED CELLS AT T=12 HR	NIL	5360	7125	3980
	+CHI	1240	3970	197
	+CHI+ER	126	343	31
CAP-TREATED CELLS AT T=24 HR	NIL	3734	6390	2570
	+CHI	1550	3670	783
	+CHI+ER	115	168	70
NORMAL CELLS AT T=12 HR	NIL	4280	7075	6250
	+CHI	171	823	34
	+CHI+ER	24	65	5

Table 3-6. *In vivo* assay of mitochondrial protein synthesis.
Cells were removed from cultures and washed. Washed cells were incubated 15 min with leucine-4-5-^{3}H (34.1 mCi/µmole, 10 µCi/100ml of cell suspension), after equilibration with antibiotics in glucose-phosphate buffer at 29°C. Incorporation was terminated by dilution of the isotope, immediate cooling of cell suspensions and centrifugation. Cells were then fractionated to obtain subcellular fractions as described in detail in Section 3-2-6. Subcellular fractions were then processed for determination of radio-activities. Results had been corrected for background but not for the efficiency of counting which was routinely about 60%. Concentrations of CHI and ER were 100 µg/ml and 5 mg/ml respectively.

CHI or EB added to individual portions of the culture. After a further 8 hr, cells were harvested and their absolute cytochrome absorption spectra were examined. Results are shown in Figure 3-11. ER added to a CAP-treated culture at late logarithmic phase (16 hr) had no apparent effect on the resynthesis of cytochromes aa_3 and *b* as cells moved into stationary phase. However, cycloheximide apparently inhibited the subsequent resynthesis of cytochromes aa_3 and *b* at stationary phase of growth. Ethidium bromide inhibited resynthesis of cytochromes aa_3 and *b* but apparently was without effect on the synthesis of cytochrome *c*.

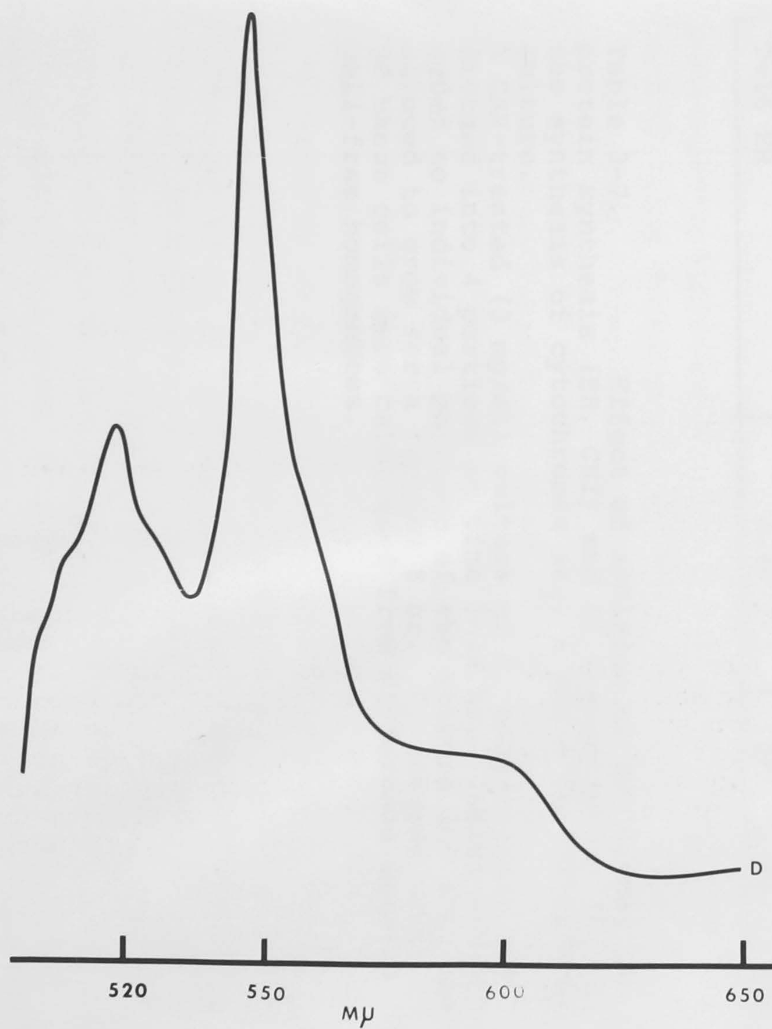
Specific cytochrome content of the cell was calculated from difference spectroscopy of the cell-free homogenate. Results are shown in Table 3-7. Erythromycin had little effect on the resynthesis of cytochromes aa_3, *b* and *c* in the chloramphenicol treated culture. Cycloheximide on the other hand, inhibited the resynthesis of all cytochromes. Ethidium bromide, a specific inhibitor of transcription of the mitochondrial genome (Zylber *et al.*, 1969) inhibited specifically the resynthesis of cytochromes aa_3 and *b* but had no effect on the synthesis of cytochrome *c*.

3-3-9 THE EFFECT OF INHIBITION BY ER, CHI AND EB ON THE SYNTHESIS OF CYTOCHROMES aa_3, *b* AND *c* IN NORMAL CULTURES

A normal culture of *Candida parapsilosis* was allowed to grow to early stationary phase (14 hr); at this time, inhibitors were added to separate aliquots of the culture and divided cultures were allowed to grow for a further 10 hr in the presence of inhibitors. Cells were then assayed for cytochromes by difference spectroscopy. As shown in Table 3-8, addition of ER had no inhibitory effect on the synthesis of all cytochromes, whereas addition of CHI or CHI + ER inhibited to a large extent the resynthesis of all cytochromes. On the other hand, ethidium bromide inhibited the synthesis of aa_3 and *b* but not that of cytochrome *c*. For comparison, a parallel experiment was carried out using *Saccharomyces cerevisiae* and results are shown in Table 3-8.

Figure 3-11. The effect of addition of ER, CHI and EB to a CAP-treated culture on the synthesis of cytochromes aa_3 and b. A CAP-treated culture of *C. parapsilosis* was divided into 4 portions at time T=16 hr and ER (5 mg/ml), CHI (100 μg/ml) or EB (100 μg/ml) was added to individual portions of the culture; one portion received no addition. Incubation of the culture was continued for a further 8 hr and then cytochrome spectra of the cells were examined. Cytochrome absorption spectra at time T=24 hr of cells treated with ER and CHI are curves B and C respectively. CAP-treated cells at time T=16 and T=24 hr had spectra D and A respectively. Cytochrome absorption spectra of cells at time T=24 hr receiving addition of EB is similar to spectrum D.

ADDITION AT T=16 HR TO CAP-TREATED CULTURES	CELL GROWTH AT T=24 HR (mg dry wt cells/ml)
(A) NIL	7.33
(B) ER (4 mg/ml)	7.33
(C) CHI (100 μg/ml)	5.40
EB (100 μg/ml)	6.40
(D) T=16 HR	4.05


D
520
550
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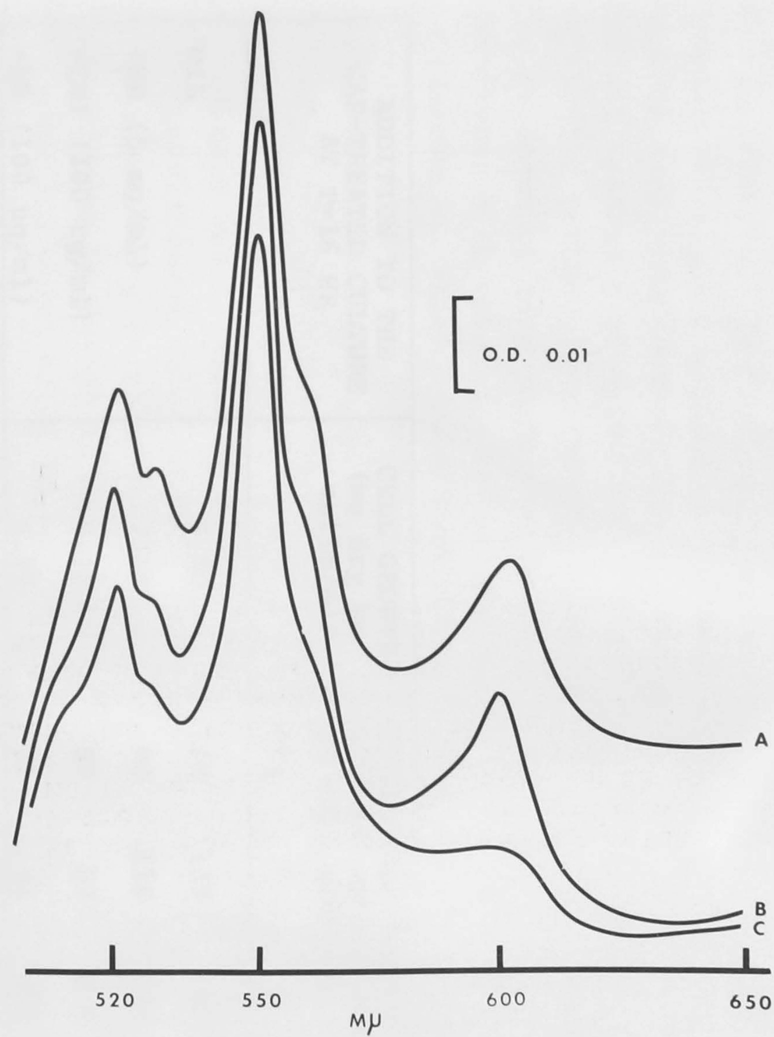
mμ


O.D. 0.01
A
B
C
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mμ

ADDITION TO THE CAP-TREATED CULTURE AT T=16 HR	CELL GROWTH (mg dry wt cells/ml)	CYTOCHROME CONTENT (mμmoles cytochrome/ gm cell protein)		
		aa_3	*b*	*c*
NIL	7.80	118	137	425
+ER (5 mg/ml)	7.60	90	115	341
+CHI (100 μg/ml)	5.10	57	82	274
+EB (100 μg/ml)	6.10	31	42	405
T=16 HR	3.40	59	90	247

Table 3-7. Effect of addition of inhibitors of protein synthesis (ER, CHI) and of transcription (EB) on the synthesis of cytochromes aa_3, *b* and *c* in CAP-treated culture.
A CAP-treated (3 mg/ml) culture of *C. parapsilosis* was divided into 4 portions at time T=16 hr. Inhibitors were added to individual portions of the culture and they were allowed to grow for a further 8 hr. Cytochrome contents of these cells were calculated from difference spectra of cell-free homogenates.

In *Saccharomyces*, ER inhibited completely the synthesis of cytochromes aa_3 and apparently had negligible effect on the synthesis of cytochromes *b* and *c*. The apparent failure to inhibit synthesis of cytochrome *b* might be ascribed to the particular method of estimating it in cell-free homogenates. The presence of very large amounts of c-type cytochromes interferes with determination of the b-type. Furthermore cytochrome b_2, which is not involved in the electron transfer chain, is present in substantial amounts in anaerobic, glucose-repressed cells or cells inhibited by CAP or ER. CHI or CHI+ER inhibited the synthesis of all cytochromes in *S. cerevisiae*. Also shown in Table 3-8, the concentration of CHI used was found to be over 90% inhibitory to whole-cell amino acid incorporation in both *Saccharomyces* and *Candida*. It is interesting to note that ER inhibited whole-cell incorporation by 40-50% in both organisms, instead of the less than 10% which might be assumed from the residual activity after CHI inhibition. This would suggest considerable interaction between mitochondrial and cytoplasmic protein synthesis, that is, protein synthesis in the CPSS being dependent on activity of the MPSS.

It appeared from experimental data that ER, when added to a normal culture of *Candida* near stationary phase of growth, had no inhibitory effect on the synthesis of cytochromes aa_3 and *b*. This observation demonstrates that the phasic phenomenon is not unique to CAP- or ER-treated cultures of *Candida* and does not depend on prior contact of cells with antibiotics. However, sensitivity towards CAP or ER could be conferred on cells at stationary phase of growth by the addition of yeast extract. Addition of yeast extract, as shown in Table 3-9, to logarithmic-phase cells enhanced the synthesis of all cytochromes. Addition of yeast extract and erythromycin specifically inhibited the synthesis of cytochromes aa_3 and *b* but not that of cytochrome *c*. The additional yeast extract increased cell yield in stationary phase, and this is consistent with the view that inhibition is seen only when cell growth is accelerated.

The data show that the loss of sensitivity towards CAP and ER of the synthesis of cytochromes aa_3 and *b* in

CELL TYPE	ADDITION AT T = 14 HR	CELL YIELD (mg dry wt/ml)	CYTOCHROME CONTENT (mμmoles cytochrome/gm cell protein)		
			aa$_3$	*b*	*c*
CANDIDA PARAPSILOSIS AT T = 24 HR	NIL	8.87	168	189	249
	+ER	10.4	178	276	286
	+ER + CHI	7.65	113	176	201
	+CHI	6.95	109	161	227
	+EB	8.50	103	154	250
AT T = 14 HR		6.55	107	154	185
SACCHAROMYCES CEREVISIAE AT T = 24 HR	NIL	6.37	68.2	176	181
	+ER	7.54	29.5	150	168
	+ER + CHI	5.20	23.0	102	102
	+CHI	4.95	21.0	150	160
AT T = 14 HR		5.00	32.0	133	135

CELL TYPE	ADDITION	WHOLE CELL INCORPORATION OF C^{14} - LEUCINE AT T = 14 HR (cpm/15 min incubation/5 mg cells)	% INHIBITION
CANDIDA PARAPSILOSIS	NIL	4952	0
	+ER	2344	53
	+ER + CHI	125	97
	+CHI	278	94
	+EB	2827	43
SACCHAROMYCES CEREVISIAE	NIL	7975	0
	+ER	4897	39
	+ER + CHI	160	98
	+CHI	317	96
	+EB	4571	43

Table 3-8. Effects of addition at time T=14 hr of ER, CHI and EB on the synthesis of cytochromes *aa*$_3$, *b* and *c* as well as amino acid incorporation by whole cells in normal cultures of *C. parapsilosis* and *S. cerevisiae*.
At time T=14 hr a normal culture of *C. parapsilosis* was divided into identical portions. Inhibitors were added to these portions of the culture at concentrations of 100 μg/ml for CHI, 5 mg/ml for ER and 100 μg/ml for EB. These separated cultures were grown for a further 10 hr and then cytochrome contents of cells of these cultures were calculated from difference spectra of cell-free homogenates.
A parallel experiment carried out with *S. cerevisiae* is also shown.
For the determination of effects of inhibitors on whole cell incorporation of C^{14}-leucine into protein, 1 ml aliquots of cultures were removed at time T=14.25 hr, 15 min after the addition of antibiotics and mixed with 0.2 ml of C^{14}-leucine (0.5 μCi/ml, 311 μCi/μmole). Incorporation was carried out at 29°C for 15 min and then cells were processed for the determination of radio-activities.

CELL TYPE	ADDITION AT T=12 HR	CELL YIELD (mg dry wt cells/ml)	CYTOCHROME CONTENT (mμmoles cytochrome/gm cell protein)		
			aa$_3$	*b*	*c*
At T=24 hr	NIL	8.94	162	177	364
	+YE	13.1	172	197	391
	+YE+ER	12.8	68	110	343
	+ER	10.0	134	169	296
At time T=12 hr		4.77	88	133	168

Table 3-9. Reconferring of sensitivity to inhibition by ER of synthesis of cytochromes *aa*$_3$ and *b* as a result of the addition of yeast extract.
A normal culture of *C. parapsilosis* was divided into 4 portions at time T=12 hr. Additions of yeast extract (2% w/v), yeast extract (2% w/v) and ER (5 mg/ml), and ER (5 mg/ml) were made to individual portions of the culture and grown for a further 12 hr. Cells of these cultures were then examined for cytochrome contents by difference spectroscopy of cell-free homogenates.

stationary phase of growth can occur in normal cultures of *C. parapsilosis*. It is not dependent on previous treatment of cells with the inhibitors during growth, and sensitivity can be re-conferred by the addition of yeast extract to stationary phase cultures. All these results thus gave positive support to the hypothesis that the loss of sensitivity to inhibitors of the MPSS in stationary phase cells is due to a dissociation or uncoupling of the interlock as a consequence of a decline in the rate of cell division. The regulatory interlock could be re-established (and with it the sensitivity to CAP and ER) by an increase in the rate of cell-division (e.g. by sub-culturing into fresh medium).

3-4 DISCUSSION

Results described in this chapter strongly suggest that the loss of sensitivity of cytochrome synthesis to CAP and ER in stationary phase cells of *C. parapsilosis* is a consequence of the metabolic state of cells in cultures, related to the potentiality for rapid cell-division and growth. These experimental observations have been interpreted in terms of the dissociation or uncoupling of an interlock between activities of the MPSS and CPSS in stationary phase cells. It is proposed that in slowly or nondividing cells (i.e. stationary phase cultures) the interlock might be less effective or even abolished, hence inhibition of the MPSS has no effect on the synthesis of cytochromes aa_3 and *b*. Obvious features of this model have been outlined and experimentally tested.

It has been reported that a nuclear mutant of *S. cerevisiae* owes its insensitivity to inhibition to CAP to a change in permeability of the cell to this inhibitor (Linnane *et al.*, 1968 a). Kellerman *et al.* (1969) have recently suggested that the partial inhibition of synthesis of cytochromes aa_3 and *b* in *C. parapsilosis* by CAP and ER is probably due to a change in permeability to these inhibitors. However, findings presented in this thesis indicate that in *Candida* the change of sensitivity towards

inhibitors of the MPSS is not a genetic event and is dependent on the cultural state. Furthermore, inhibitors of mitochondrial protein synthesis tested are very different chemically, and the phasic character of the phenomenon does not extend to an inhibitor of the transcription process (ethidium bromide). Data on the uptake of CAP by cells clearly indicate that changes in permeability cannot explain the phenomenon. The partial inhibition of synthesis of cytochromes aa_3 and *b* in *C. parapsilosis* as reported by Kellerman *et al.*, may be explained almost certainly in terms of their cells having been in the process of resynthesis of cytochromes.

In procaryotes, such as *Staphylococcus aureus* and *Escherichia coli*, many mutants exhibit resistance towards inhibition by inhibitors of protein synthesis such as CAP by virtue of an enzymatic capacity to conjugate or acetylate and hence inactivate them (Shaw and Brodsky, 1967). The duration of the lag phase of growth of these bacteria was proportional to the external concentration of CAP. The lag period represented the time required for the production of acetylating enzyme and the inactivation of CAP to a level permitting cell growth. This "phasic phenomenon" in bacteria might be taken to explain the phasic response as observed in *C. parapsilosis* with respect to the synthesis of cytochromes aa_3 and *b*. Again, it is difficult to accept this as being particularly likely when similar effects are observed with ER, a macrolide antibiotic, quite different in chemical structure and mode of action from CAP (Weisblum and Davies, 1968). This suggests that if enzymic inactivation did occur, the responsible enzyme might either be very low in specificity, or there would be multiple inactivating enzymes each specific for one of several antibiotics. The latter explanation appears unlikely because once the cell becomes insensitive to CAP, further addition of ER does not elicit further inhibition. Direct assay of culture medium for unconjugated CAP demonstrated that there was no substantial loss.

Mutants of *S. cerevisiae* have recently been described which have modified mitochondrial protein synthesising systems such that *in vivo* synthesis of cytochromes aa_3 and b is no longer sensitive to inhibition by ER (Linnane *et al.*, 1968 b; Thomas and Wilkie, 1968). It is possible that a similar modification of the MPSS is the mechanism whereby stationary phase cells of *C. parapsilosis* gained their apparent insensitivity towards CAP and ER, and could therefore synthesise cytochromes aa_3 and b in the presence of inhibitors. The sensitivity of the MPSS towards inhibitors was examined by both *in vitro* and *in vivo* techniques. The *in vivo* technique described by Schatz and Saltzgaber (1969) was adopted because it could be argued that results obtained from experiments with isolated mitochondria are inherently unreliable due to possible artifacts generated by isolation. Thus, it is conceivable that the MPSS in stationary phase cells of *C. parapsilosis* is insensitive to ER or CAP *in situ* but is rendered sensitive on isolation. Both *in vivo* and *in vitro* results showed that there was no apparent change in sensitivity of the MPSS in stationary phase cells towards ER and CAP.

The reversibility of the phasic phenomenon argues against explanations in terms of permanent genetic or major physiological changes, such as the induction of antibiotic-inactivating enzymes, development of cellular or organelle impermeability towards antibiotics, and persistent modifications of the MPSS.

Strong evidence against these possibilities was the demonstration that the phasic phenomenon in stationary phase occurred naturally in normal cultures and was therefore independent of previous exposure of cells to antibiotics.

To recapitulate, because the phasic phenomenon could be demonstrated against both chloramphenicol and erythromycin, which are very different in chemical structure and mode of action, the phenomenon is probably a physiological response. Genetic change as the basis of the phenomenon is made unlikely by the reversibility and environmental control of the phenomenon. Also, the phenomenon was seen in cells previously unexposed to antibiotics.

The "dissociation-association of interlock hypothesis" is strongly supported by the results of tests. However, it should be pointed out that this hypothesis needs to be tested on other organisms. Also, no test has been made of the possibility of a switch-over of the site of synthesis of cytochromes aa_3 and *b* from the MPSS in the logarithmic phase cells to the CPSS in the stationary phase cells. This although conceivable, would be extremely difficult to test experimentally.

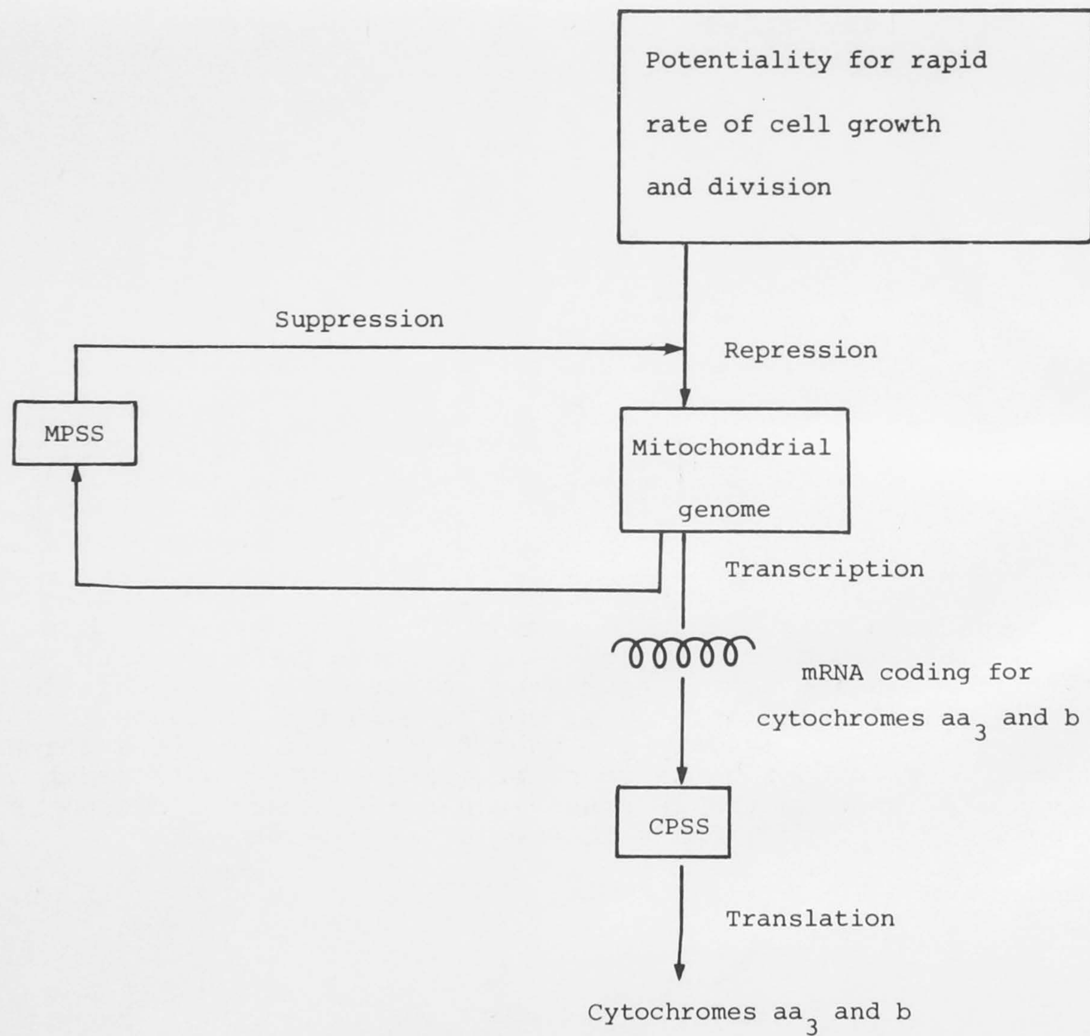
The molecular mechanism of the interlock remains speculative. Indeed, the whole question of inter-regulatory relationship of mitochondrial and cytoplasmic protein syntheses, though established is characterised by a lack of precise models capable of being experimentally tested.

Up to the present time, the site of synthesis of cytochromes aa_3 and *b* remained an open question (see review by Ashwell and Work, 1970). The hypothesis described in this thesis specifies the site of synthesis to be the CPSS. This is substantiated by the synthesis of cytochromes aa_3 and *b* in the presence of CAP and ER, and by the inhibition of synthesis of cytochromes aa_3 and *b* by CHI in stationary phase cells. Furthermore, the particular characteristic of *C. parapsilosis* in stationary phase of growth in being no longer dependent upon activity of the MPSS for the synthesis of cytochromes aa_3 and *b*, the origin of the genetic information for the synthesis of cytochromes aa_3 and *b* might be meaningfully inferred from studies using ethidium bromide. EB is generally accepted as an inhibitor of transcription specific for mitochondrial genomes (Zylber *et al.*, 1969). Ethidium bromide inhibited preferentially the synthesis of cytochromes aa_3 and *b* in stationary phase cells of *C. parapsilosis*, but had no inhibitory effect on the synthesis of cytochrome *c*, which is generally thought to be coded by nuclear genes (Roodyn and Wilkie, 1968). Based on the accepted mode of action of EB, it can be concluded that cytochromes aa_3 and *b* are coded by the mitochondrial genome.

If information for cytochromes aa_3 and *b* were transcribed from the mitochondrial genome and translated by the CPSS, the immediate question would be, what is the role of the

MPSS in the hypothetical interlock between the two systems governing the synthesis of cytochromes aa_3 and *b*. Closer analysis of the proposed hypothesis allows an answer to the question of a possible role for the MPSS to be found. Essentially the proposed hypothesis states that, through the intervention or participation of the MPSS, cellular synthesis of cytochromes aa_3 and *b* in the CPSS is co-ordinated with cellular growth and division, and when the cellular tendency to grow and divide subsides, synthesis of cytochromes aa_3 and *b* continues in the CPSS but does not depend on the active participation of the MPSS. Thus, the MPSS apparently plays a regulatory role in the synthesis of cytochromes aa_3 and *b*. A speculative scheme for such regulation is shown in the diagram (Fig. 3-12). The mitochondrial genome generates transcripts which are translated in the CPSS to produce cytochromes aa_3 and *b*. Transcription of the mitochondrial genome coding for cytochromes is repressed by the potentiality for rapid rate of cell growth and division. This repression is however suppressed by a protein product of the MPSS. Normally, when the tendency for cell growth and division is high, repression of mitochondrial transcription is severe, but this repression is counter-acted by a suppressor synthesised by the MPSS allowing synthesis of cytochromes aa_3 and *b* (on which *Candida* is obligately dependent). Inhibition of the MPSS by CAP or ER results in an inhibition of synthesis of the suppressor, hence effectively enhances the repression (by eliminating the suppressor) and thus indirectly inhibits synthesis of cytochromes aa_3 and *b*. For balanced metabolism the activity of the MPSS in producing the suppressor may also be controlled. When the potentiality for rapid growth is low, repression of transcription of mitochondrial genomes may be negligible and structural genes for cytochromes aa_3 and *b* become fully activated. Synthesis of cytochromes aa_3 and *b* can continue in the CPSS in the presence of inhibitors of the MPSS even though the suppressor is eliminated by the inhibition of the MPSS. By translating the proposed hypothesis into molecular terms, a plausible mechanism for

Figure 3-12. A speculative scheme of a regulatory role played by the MPSS. Mitochondrial genome contains structural genes of cytochromes aa_3 and *b* as well as a suppressor. Expression of structural genes of the cytochromes is repressed by the potentiality for rapid rate of cell growth and division. This repressive control of transcription of genes of the cytochromes is however counteracted by a suppressor synthesized by the MPSS. By means of this interaction between repressor and suppressor, transcription of genes of the cytochromes (hence synthesis of cytochromes which takes place in the CPSS) is allowed even when the potentiality for rapid cell growth is high, i.e. in logarithmic phase of normal cultures. Thus, the role played by the MPSS is the synthesis of a suppressor. In logarithmic phase of growth, inhibition of the MPSS by CAP or ER eliminates the suppression, and therefore effectively accentuate the repression resulting in the inhibition of synthesis of the cytochromes. In stationary phase of growth (or nondividing cells), repression of transcription of the mitochondrial genome is negligible or nonexistent, absence or presence of the suppressor has no overall effect on the synthesis of the cytochromes.



Potentiality for rapid
rate of cell growth
and division
Suppression
Repression
MPSS
Mitochondrial
genome
Transcription
mRNA coding for
cytochromes aa_3 and b
CPSS
Translation
Cytochromes aa_3 and b

the interlock between the MPSS and CPSS is provided. The interlock is actually the suppression by a protein product of the MPSS of the repression of the transcription of mitochondrial genomes by the metabolic state of the cell.

CHAPTER 4

A COMPARATIVE STUDY OF THE MACROMOLECULAR COMPONENTS OF THE MPSS OF *C. PARAPSILOSIS* AND *S. CEREVISIAE* (1) MITOCHONDRIAL RIBOSOMES AND RNA OF *C. PARAPSILOSIS*.

CHAPTER 4

4-1 INTRODUCTION

In the previous two chapters, I have discussed the respiratory physiology of the obligate aerobe, *Candida parapsilosis*, comparing it with *Saccharomyces cerevisiae*. Certain interesting features have emerged from this comparative study and these have been discussed. *C. parapsilosis* and *S. cerevisiae* differ in a further fundamental aspect of mitochondrial differentiation. In the Introduction to this thesis (Chapter 1) I mentioned that mitochondrial differentiation in *S. cerevisiae* is known to be controlled by genetic determinants, in particular extrachromosomal genes. Cytoplasmically-inherited, respiratory-deficient mutants of *S. cerevisiae* occur with high frequency, and the frequency of extrachromosomal mutation may be enhanced greatly by ethidium bromide without affecting nuclear genes. Mutants that result, apart from being respiratory-incompetent and lacking cytochromes aa_3 and b, have altered buoyant density of their mitochondrial DNA and apparently have lost their mitochondrial protein synthesising activity. In obligate aerobes, cytoplasmically-inherited, respiratory-deficient mutants are rare and generally unstable (Bulder, 1964 a,b; Heslot *et al.*, 1970 a,b). *C. parapsilosis* treated with ethidium bromide does not appear to generate respiratory-deficient mutants (Kellerman *et al.*, 1969). When *C. parapsilosis* is cultured in the presence of ethidium bromide, synthesis of cytochromes aa_3 and b is inhibited (see Chapter 2; also Kellerman *et al.*, 1969). These cytochrome-deficient cells are not respiratory-incompetent, they have respiratory activities of the same magnitude as those of normal cells. However, their respiratory activities are completely insensitive to cyanide. Effects of ethidium bromide on *C. parapsilosis* are reversible. On subculturing ethidium bromide-treated cells into fresh medium without ethidium bromide, cells synthesize

cytochromes *aa*$_3$ and *b* normally (Kellerman *et al.*, 1969). It therefore appeared that another source of fundamental difference might be found in the extrachromosomal genetic apparatus of these two organisms, in particular, macromolecular constituents such as DNA and RNA. In this and the following chapter, I propose to describe experiments aimed at examining the macromolecular constituents of the mitochondrial protein synthesising system of these two organisms. These systems appear to be very similar in terms of their overall activity, their energy requirement, and their sensitivity towards inhibitors (see Chapter 3). I shall deal with *C. parapsilosis* in this chapter, and *S. cerevisiae* in chapter 5.

4-2 EXPERIMENTAL

4-2-1 ^{32}P-LABELLING OF RNA

Cells in liquid culture were grown to 10 hr and 5 mCi of carrier-free ^{32}P-orthophosphate (sodium salt, pH7) per litre of culture were then added. In the case of ethidium bromide treatment, the ^{32}P-orthophosphate was added 5 min after the culture was treated with 100 µg/ml of ethidium bromide (EB). Control and EB-treated cultures were incubated for 6 hr in the presence of the isotope. Immediately prior to cell fractionation equal weights of unlabelled carrier cells, grown for the same time were mixed with both the labelled control and labelled EB-treated cells to provide an optical density marker for the RNA species.

4-2-2 DISRUPTION OF CELLS AND PREPARATION OF SUBCELLULAR FRACTIONS

Cells were harvested by centrifugation at 0-4°C, washed twice with distilled water, and once with sucrose-AMT buffer (0.5M sucrose, 0.1M ammonium chloride, 0.01M magnesium chloride, 0.01M Tris-HCl pH 7.6, 0.01M 2-mercaptoethanol). Cells were then resuspended in an equal volume of sucrose-AMT buffer, mixed with 1 volume of glass beads (diameter 0.45-0.5mm) per volume of cell suspension,

and broken in a Braun MSK homogenizer at maximum speed for 25 sec, with carbon dioxide as coolant, as described by Schatz (1967). The brei was decanted and glass beads were rinsed three times with one volume of sucrose-AMT buffer. The brei and washings were pooled and centrifuged twice at 3000 rpm for 10 min (GS3, Sorvall) to remove nuclei and cell debris. The cell-free homogenate from the second 3000 rpm centrifugation was then centrifuged at 9000 rpm for 20 min (Sorvall, SS34) to sediment a crude mitochondrial fraction. The supernatant is designated *postmitochondrial supernatant*.

4-2-3 PURIFICATION OF MITOCHONDRIAL FRACTION

The crude mitochondrial pellet was resuspended in about 3 volumes of sucrose-AMT buffer and purified by methods A, B or C, described below.

Method A: Crude mitochondrial suspension (5 ml containing not more than 100 mg protein) was layered over a two-step discontinuous sucrose gradient of 10 ml each of 30% (w/v) and 40% (w/v) sucrose in AMT buffer, and centrifuged in a SW 25.1 rotor (Beckman) at 25,000 rpm for 1 hr at 4°C to give a partially purified mitochondrial pellet.

Method B: Mitochondrial suspension was layered over a 4-step discontinuous sucrose gradient consisting of 5 ml each of 85%, 60%, 40% and 30% (w/v) sucrose in AMT buffer. Gradients were centrifuged at 25,000 rpm (SW 25.1 rotor, Beckman) for 1 hr at 4°C. Mitochondria banded at the 40%-60% sucrose interface under these conditions. The mitochondrial band was removed and the osmolarity reduced to approximately 0.5M by mixing with 2 volumes of AMT buffer. The mitochondrial suspension was then layered over a 2-step discontinuous sucrose gradient as described in Method A.

Method C: The method is similar to Method B except that an SW 27 rotor (Beckman) was used; the 4-step discontinuous sucrose gradient was composed of 5 ml of 85%, 15 ml of 60%, 5 ml of 40% and 5 ml of 30% (w/v) sucrose. Banding of mitochondria at the 40%-60% interface was achieved by

centrifugation at 17,500 rpm for 1 hr at 4°C. The mitochondrial band was removed as described in Method B and then pelleted through a 2-step gradient as in Method A, by centrifuging at 27,000 rpm (SW 27 rotor) for 1 hr at 4°C.

4-2-4 PREPARATION OF CYTOPLASMIC RIBOSOMES

The postmitochondrial supernatant, obtained from the 9000 rpm centrifugation of the cell-free homogenate, was treated with Triton X-100 (final concentration 5% v/v), and centrifuged at 16,000 rpm for 10 min (Sorvall, SS34) to remove large particulate and membranous material. The clarified lysate was centrifuged at 50,000 rpm (Beckman, 50 rotor) for 1 hr at 2°C to sediment cytoplasmic ribosomes. Pellets were resuspended in AMT buffer for loading onto linear sucrose gradients.

4-2-5 PREPARATION OF RIBOSOMAL PARTICLES FROM PURIFIED MITOCHONDRIA

Purified mitochondrial pellets were resuspended in AMT buffer at a concentration of approximately 10 mg protein per ml. Triton X-100 was added at a protein : Triton X-100 ratio of 1:5 (w/w) to lyse mitochondrial membranes. Solubilization was assisted by homogenization using a syringe with 22G needle, then left at 0° for 20 min. The lysate was clarified by centrifugation at 16,000 rpm for 10 min to remove insoluble material (Sorvall, SS34). Ribosomal particles were pelleted from the lysate by centrifuging at 50,000 rpm (Beckman, 50 rotor) for 1 hr at 2°C. Translucent, gelatinous pellets (ribosomal particles) were resuspended in AMT buffer for loading onto continuous sucrose gradients as described below (Section 4-2-6).

Recovery of RNA-containing material after lysis of mitochondrial fraction (containing 30-40 μg RNA/mg protein) with Triton X-100 is shown in the following Table.

Fraction	RNA recovered
lysate (insoluble material + clarified lysate)	1200 μg
insoluble material	170
clarified lysate	770
ribosomal particles	650
post-ribosomal supernatant	120

4-2-6 SUCROSE DENSITY GRADIENT ANALYSIS OF RIBOSOMAL PARTICLES

Ribosomal pellets resuspended in 1 ml of AMT buffer were applied to 36 ml linear sucrose density gradients (10%-30% (w/v) sucrose in AMT buffer). Gradients were centrifuged at 27,000 rpm (Spinco, SW 27 rotor) for 4 hr at $4^{o}C$. The distribution of ribosomes in gradients were measured at 254 nm with an ISCO density gradient fractionator with 2 mm light-path flow cell.

4-2-7 EXTRACTION OF RNA

The method used for the isolation of RNA from subcellular fractions is a modification of the sodium deoxycholate-phenol method described by Click and Hackett (1966). Mitochondrial pellets were resuspended in 10 ml of buffered deoxycholate (0.1M sodium glycinate pH 8.0, 0.1M sodium chloride, 0.01M sodium EDTA, 1% (w/v) sodium deoxycholate and 1% (w/v) bentonite) and homogenized for 2 min in a Braun shaker at low speed setting with carbon dioxide as coolant. 25 ml of phenol saturated with an aqueous solution of 1mM EDTA was added to the homogenate, and the mixture was shaken at 400 cycles/min for 10 min at $4^{o}C$. The mixture was centrifuged at 11000 rpm for 5 min (Sorvall, SS34) to resolve the aqueous and phenol phase. The aqueous phase was removed and extracted twice with ice-cold anhydrous diethyl ether. Two volumes of cold 95% ethanol containing 10mM sodium acetate were added to the aqueous phase, and cooled to -20° for 18 hr to

precipitate RNA. RNA was recovered by centrifugation at 13000 rpm for 30 min (Sorvall, SS34). The nucleic acid pellet was dissolved in electrophoresis buffer (described below) containing 20% (w/v) sucrose.

4-2-8 POLYACRYLAMIDE GEL ELECTROPHORESIS OF RNA

The RNA was subjected to polyacrylamide gel electrophoresis using the Tris-acetate-EDTA buffer system containing 0.1% sodium dodecyl sulphate described by Loening (1969). 2.4% polyacrylamide gels of 9 cm length were prerun for 30 min before use. The RNA sample (10-30 µl, containing 20-60 µg RNA in electrophoresis buffer - 20% (w/v) sucrose) was applied and electrophoresis was continued for 2 or 4 hr at 22°C and 5mA per gel. Gels were then removed from plexiglass tubes and soaked in water for 1 hr. Absorption profiles were measured at 265 nm using a Joyce-Loebl Chromoscan.

4-2-9 PREPARATION OF POLYACRYLAMIDE GELS FOR RADIO-ACTIVITY DETERMINATIONS

Washed gels were frozen on solid carbon dioxide and cut into 1 mm slices with a Mickle gel slicer. Gel slices were hydrolyzed in 0.5 ml of 10% (v/v) piperidine containing 1mM EDTA by heating at 65°C for 4 hr. Dried slices were then allowed to swell for 2 hr in 0.5 ml water before adding 10 ml of toluene-Triton X-100 scintillant (Triton X-100, 33% (v/v); PPO, 0.2% (w/v); POPOP, 0.02% (w/v); in toluene) and counted.

4-2-10 BASE COMPOSITION OF RNA

Samples of RNA were hydrolyzed in alkali and resulting nucleoside monophosphates were separated quantitatively as described by Katz and Coomb (1963).

4-2-11 THERMAL DENATURATION OF RIBOSOMES

After separation on linear sucrose gradients as described above, purified mitochondrial and cytoplasmic

ribosomes were diluted into AMT to an optical density at 260nm (10 mm light path) of 0.2 - 0.3. 3.8 ml of these suspensions were sealed in quartz cuvettes (4 ml, 10 mm light path) and optical density readings taken automatically at 20 sec intervals as the temperature of the cell carrier of the spectrophotometer (Zeiss PMQ II) was increased by approximately $1^{o}C$/min. The temperature of the cell content was recorded using a calibrated thermistor inserted in a cell containing buffer only. At the end of experiments, cuvettes were weighed and compared with their weights prior to heating. Evaporative losses were less than 1.0%. Results are not corrected for the thermal expansion of water.

4-2-12 DIGESTION OF NUCLEIC ACID EXTRACTS WITH NUCLEASES

(i) Ribonuclease digestion

Ethanol-precipitated nucleic acids were redissolved in electrophoresis buffer (Tris-acetate 40 mM, sodium acetate 20 mM pH7.8, sodium EDTA 1mM) mixed with 0.5 mg per ml of pancreatic ribonuclease (Sigma), and incubated for 5 min at 37°. After digestion, aliquots of the mixture were applied to gels and analyzed by electrophoresis.

(ii) Deoxyribonuclease digestion

Ethanol precipitates of nucleic acid extracts were dissolved in 0.75 ml of electrophoresis buffer containing 30 mM magnesium sulphate, and deoxyribonuclease (ribonuclease-free, Sigma) was added to a final concentration of 10 µg/ml. Digestion was carried out at $37^{o}C$ for 5 min. After digestion, aliquots of the mixture were applied to gels and analyzed by electrophoresis.

4-3 RESULTS

In experiments described in this chapter and the next a mechanical method of cell disruption has been used. In certain respects this method has disadvantages, for example in the extent of apparent contamination of mitochondria with cytoplasmic ribosomes. However, advantages offered outweigh this particular problem, which can be controlled,

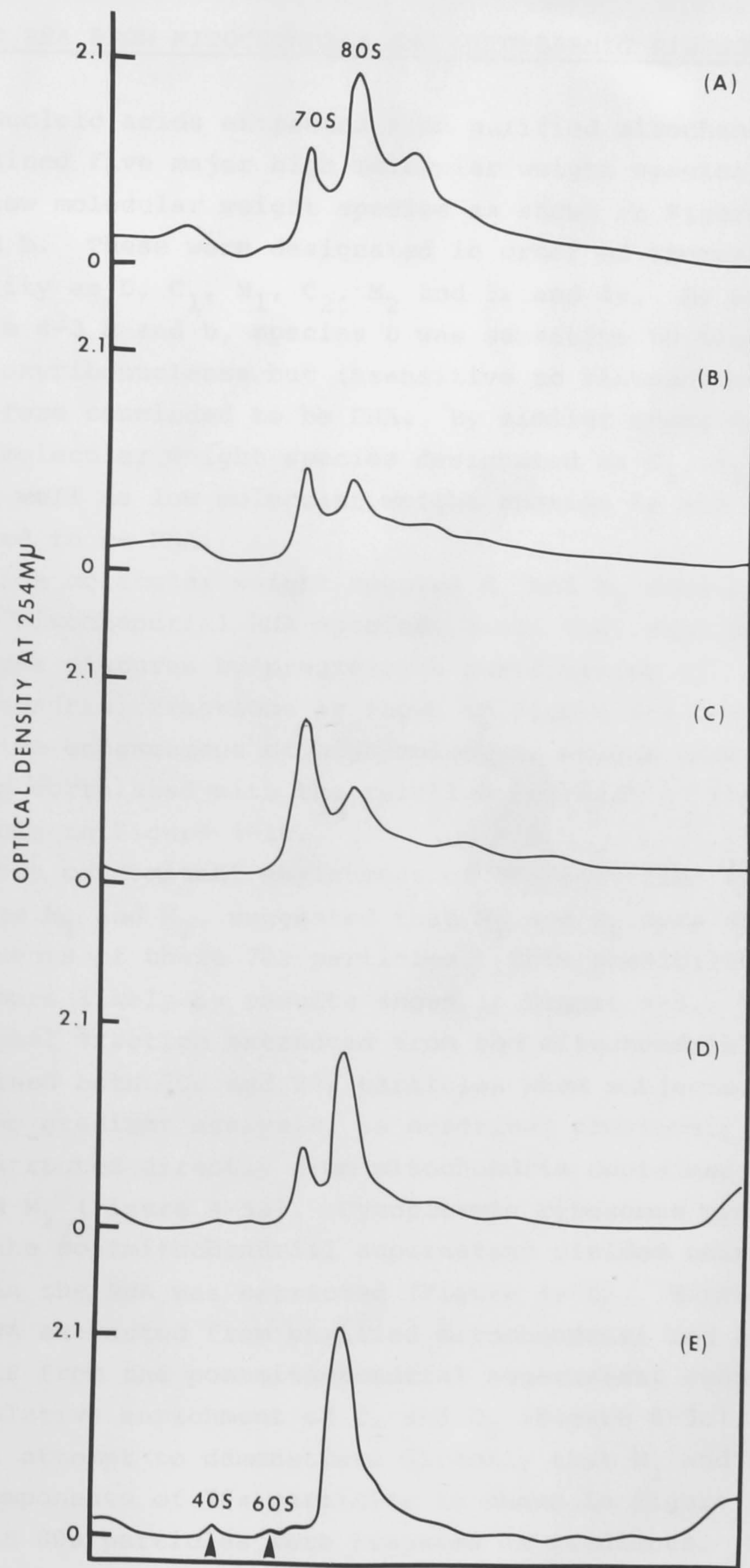
though not completely eliminated, by using a more extensive purification procedure than is required when similar preparations are made from protoplasts. Major advantages lie in the speed with which cells are broken and processed, and in the low temperature used. These are important considerations in labelling experiments since the immediate fate of the incorporated isotope can be more readily assessed. Minor advantages lie in the economy and scale with which the mechanical method can be used. Functional properties of mitochondria prepared in this way compare favourably with those of mitochondria prepared from protoplasts.

4-3-1 ISOLATION OF RIBOSOMES

Ribosomes extracted after lysis of crude mitochondrial preparations with detergent and sedimented through linear sucrose gradients resolved into two major peaks (Figure 4-1a). Calibration of these gradients with cytoplasmic ribosomes and ribosomal subunits from *S. cerevisiae*, and with ribosomes from *Escherichia coli*, indicated that ribosomes isolated from these mitochondrial preparations had sedimentation co-efficients of 70*s* and 80*s*. 80*s* particles were considered to represent contamination by cytoplasmic ribosomes, since a progressive reduction of the proportion of 80*s* particles was achieved as purification of the mitochondrial fraction proceeded (Figure 4-1 b and c). Furthermore, the addition of cytoplasmic ribosomes to preparations of mitochondrial ribosome resulted in augmentation of the 80*s* peak (Figure 4-1 d). The postmitochondrial supernatant contained 80*s* particles and trace amounts of 60*s* and 40*s* subunits (Figure 4-1 e).

The use of mitochondrial purification procedures which gave preparations of the type illustrated in Figure 4-1 c, resulted in samples contaminated about 30% by 80*s* particles. As judged by the purification achieved by the two discontinuous sucrose gradient centrifugation procedures (compare Figures 4-1 a, b and c), it is likely that even less contaminated preparations of 70*s* particles could probably be achieved by incorporating further appropriate procedures into the purification scheme.

Figure 4-1. Sedimentation patterns of mitochondrial and cytoplasmic ribosomal preparations. Ribosomal preparations extracted from mitochondrial fractions using Triton X-100 (Section 4-2-5) and from the PMS (Section 4-2-4) were analyzed by centrifugation at 27000 rpm for 4 hr (Spinco, SW 27) in sucrose density gradients, 10-30% (w/v) in AMT buffer (NH_4Cl 0.1M, $MgCl_2$ 0.01M, Tris-HCl 0.01M pH 7.6). The distribution of ribosomal particles in gradients was measured with an ISCO density gradient fractionator. Direction of sedimentation is from left to right. (A), (B), and (C) are sedimentation patterns of ribosomal preparations extracted from mitochondrial fractions purified by methods A, B and C (Section 4-2-3) respectively. (E) is the sedimentation pattern of cytoplasmic ribosomal preparation and (D) is a mixture of mitochondrial (method C) and cytoplasmic ribosomal preparations. In each case, 16-18 $O.D._{260\ m\mu}$ units was applied to the gradient.



OPTICAL DENSITY AT 254MJ
2.1
0
80S
70S
(A)
(B)
(C)
(D)
(E)
40S
60S

4-3-2 RNA FROM MITOCHONDRIA AND CYTOPLASMIC RIBOSOMES

Nucleic acids extracted from purified mitochondria contained five major high molecular weight species and two low molecular weight species as shown in Figure 4-2 a and b. These were designated in order of increasing mobility as D, C_1, M_1, C_2, M_2 and 5*s* and 4*s*. As shown in Figure 4-3 a and b, species D was sensitive to digestion by deoxyribonuclease but insensitive to ribonuclease, and therefore concluded to be DNA. By similar experiments, high molecular weight species designated as C_1, M_1, C_2, and M_2 as well as low molecular weight species 5*s* and 4*s* were defined to be RNA.

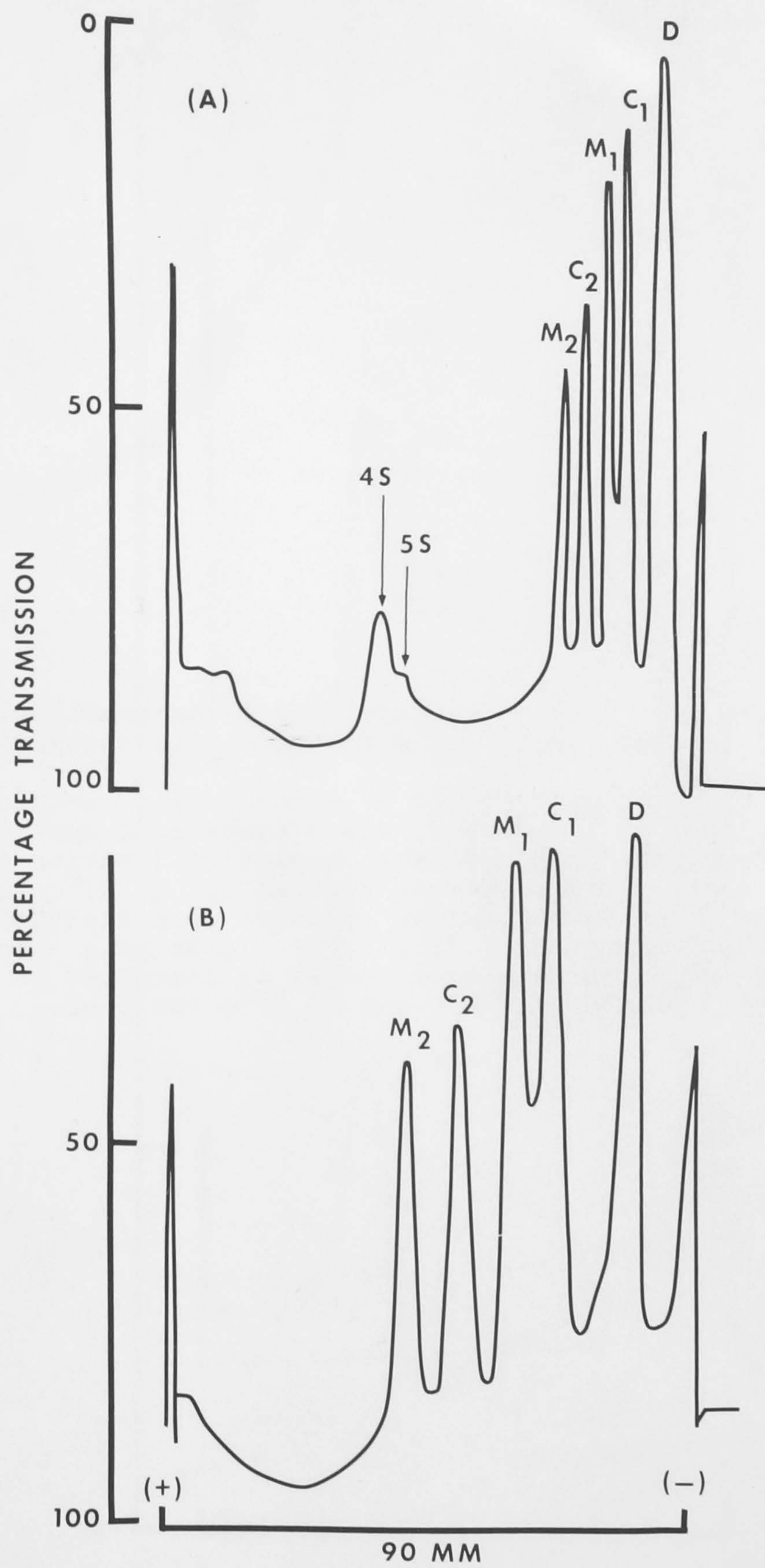
High molecular weight species M_1 and M_2 were considered to be mitochondrial RNA species, since they were enhanced in these mixtures by progressive purification of mitochondrial fractions as shown in Figure 4-4 a and b. The relative enhancement of high molecular weight species M_1 and M_2 was correlated with the relative increase of the 70*s* particle in Figure 4-1c.

This concomitant enrichment of 70*s* particles and RNA species M_1 and M_2, suggested that M_1 and M_2 were RNA components of these 70*s* particles. This possibility was made more likely by results shown in Figure 4-5. The ribosomal fraction extracted from the mitochondrial fraction contained both 70*s* and 80*s* particles when subjected to sucrose gradient analysis, as described previously, and the RNA extracted directly from mitochondria contained C_1, M_1, C_2 and M_2 (Figure 4-5a). Cytoplasmic ribosomes prepared from the postmitochondrial supernatant yielded only C_1 and C_2 when the RNA was extracted (Figure 4-5b). Mixtures of the RNA extracted from purified mitochondrial and high speed pellets from the postmitochondrial supernatant resulted in the relative enrichment of C_1 and C_2 (Figure 4-5c).

An attempt to demonstrate directly that M_1 and M_2 were RNA components of 70*s* particles is shown in Figure 4-6a. 70*s* and 80*s* particles were prepared on gradients. These ribosomal species were separated, and precipitated with ethanol at $-20^{\circ}C$ for 16 hr. RNA was then extracted from ethanol precipitates and analysed by gel electrophoresis.

Figure 4-2. Nucleic acid extracts of mitochondria of *C. parapsilosis*.

Nucleic acids extracted by phenol-SDS (Section 4-2-7) from a mitochondrial fraction purified by method B of Section 4-2-3 were analyzed by electrophoresis in 2.4% polyacrylamide gels using the Tris-acetate-EDTA buffer system containing 0.1% sodium dodecyl sulphate (SDS) described by Loening (1969). Direction of migration is from right to left. The nucleic acid sample was subjected to electrophoresis for 2 hr (A) and 4 hr (B) at 5 mA per gel at 22°C. Absorption profiles were measured at 265 mμ using a Joyce-Loebl Chromoscan.



PERCENTAGE TRANSMISSION
0
50
100
(A)
D
C1
M1
C2
M2
4S
5S
(B)
M1
C1
D
C2
M2
50
100
(+)
(−)
90 MM

Figure 4-3. Sensitivity of macromolecules in nucleic acid extract of mitochondria to digestion by nucleases.

The nucleic acid extract of mitochondria, obtained as described in Figure 4-2, was dissolved in appropriate buffers for RNase and DNase digestion (Section 4-2-12 (i) and (ii)). Digestion was carried out at 37°C for 5 min. After digestion, reaction mixtures were analyzed by electrophoresis in 2.4% polyacrylamide gels using the Tris-acetate-EDTA buffer system as described in Section 4-2-8. Electrophoresis was at 22°C for 2 hr.

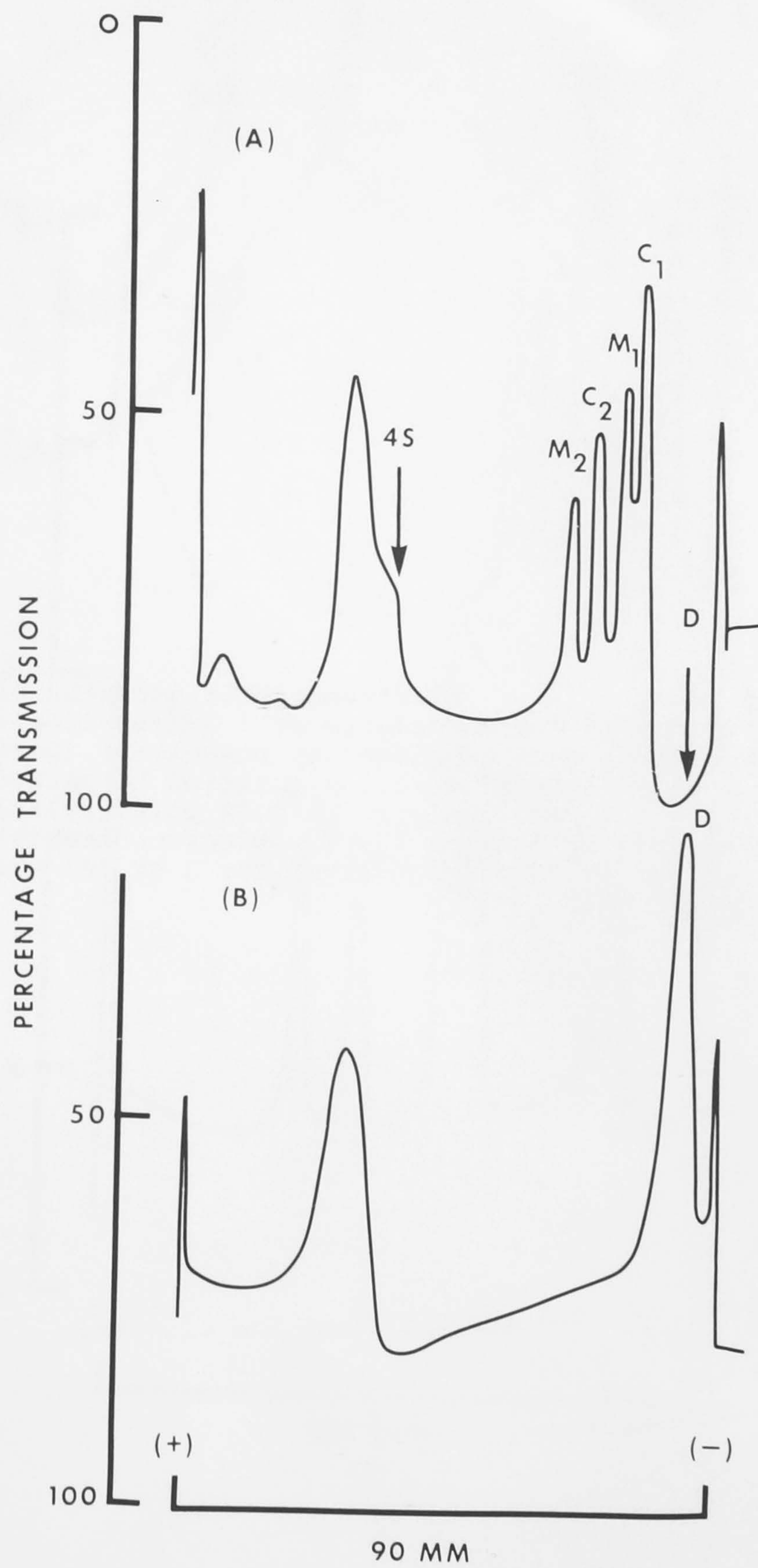

O
50
100
(A)
4S
M2
C2
M1
C1
D
D
PERCENTAGE TRANSMISSION
(B)
50
100
(+)
(−)
90 MM

Figure 4-4. Electrophoretic patterns of nucleic acid extracts of mitochondria of *C. parapsilosis*. Nucleic acids were extracted by phenol-SDS (Section 4-2-7) from a mitochondrial fraction purified by method C of Section 4-2-3, and analyzed in 2.4% polyacrylamide gels as described in Section 4-2-8. Electrophoresis was carried out at room temperature for 2 hr (A) and 4 hr (B) at 5mA per gel.

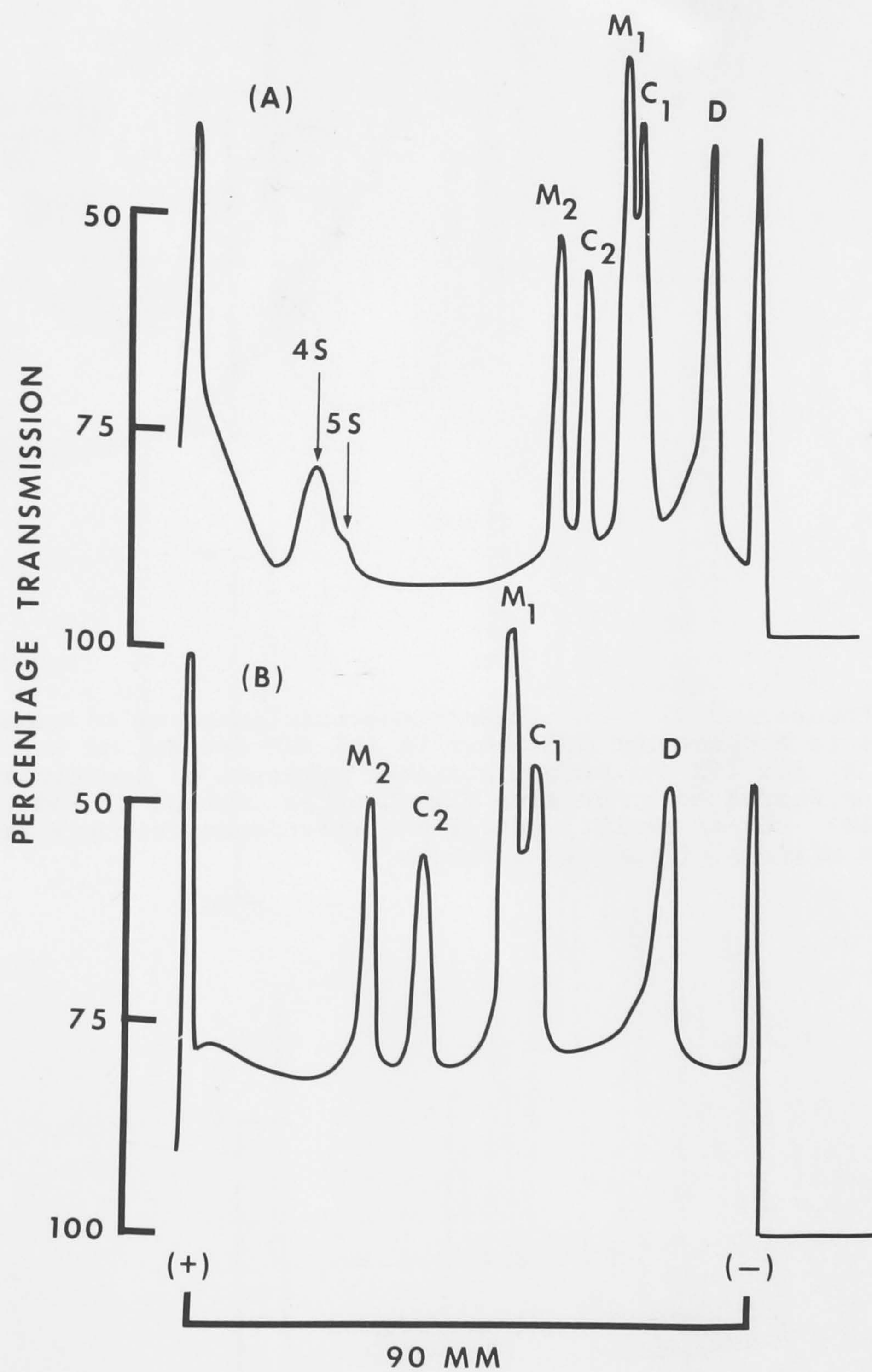

(A)
M_1
C_1
D
M_2
C_2
4S
5S
50
75
100
PERCENTAGE TRANSMISSION
(B)
M_1
C_1
M_2
C_2
D
50
75
100
(+)
(−)
90 MM

Figure 4-5. Electrophoretic patterns of nucleic acid extracts of mitochondria and RNA extract of the PMS. (A) and (B) are electrophoretic patterns of nucleic acids extracted from purified mitochondria (method C) and the PMS respectively. (C) is the electrophoretic pattern of a mixture of the two extracts.

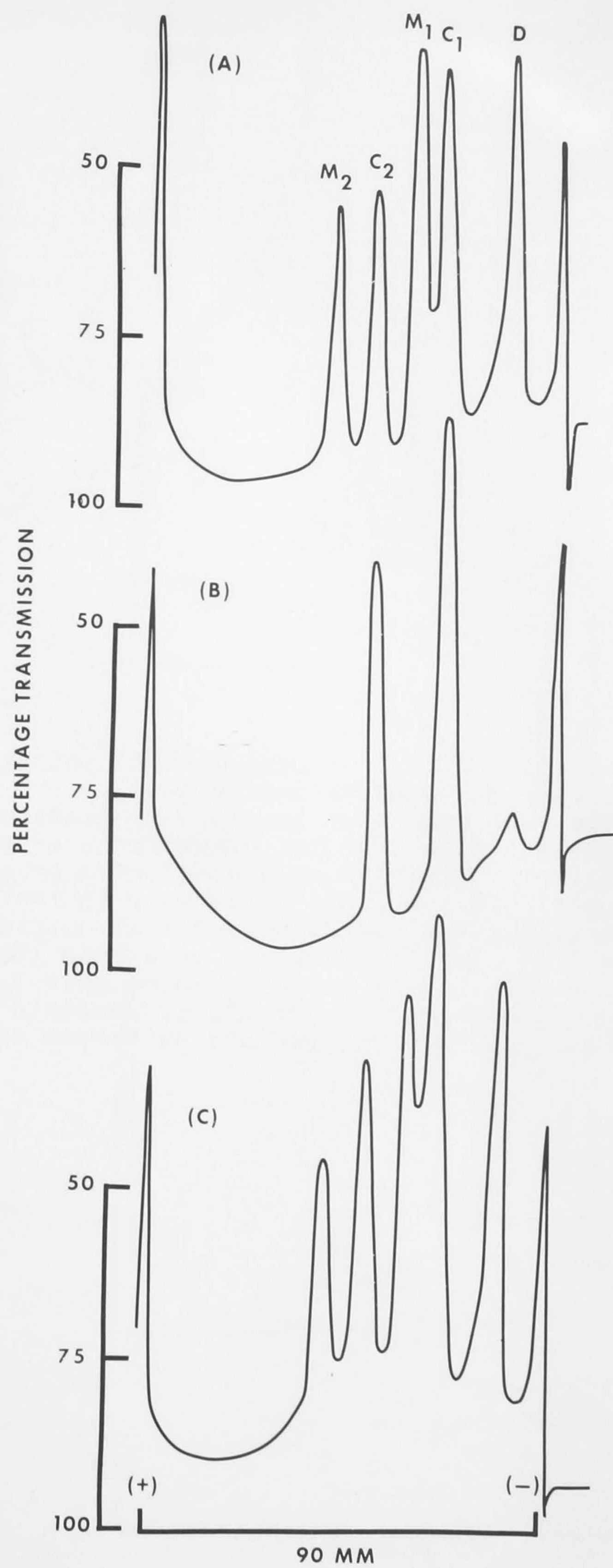
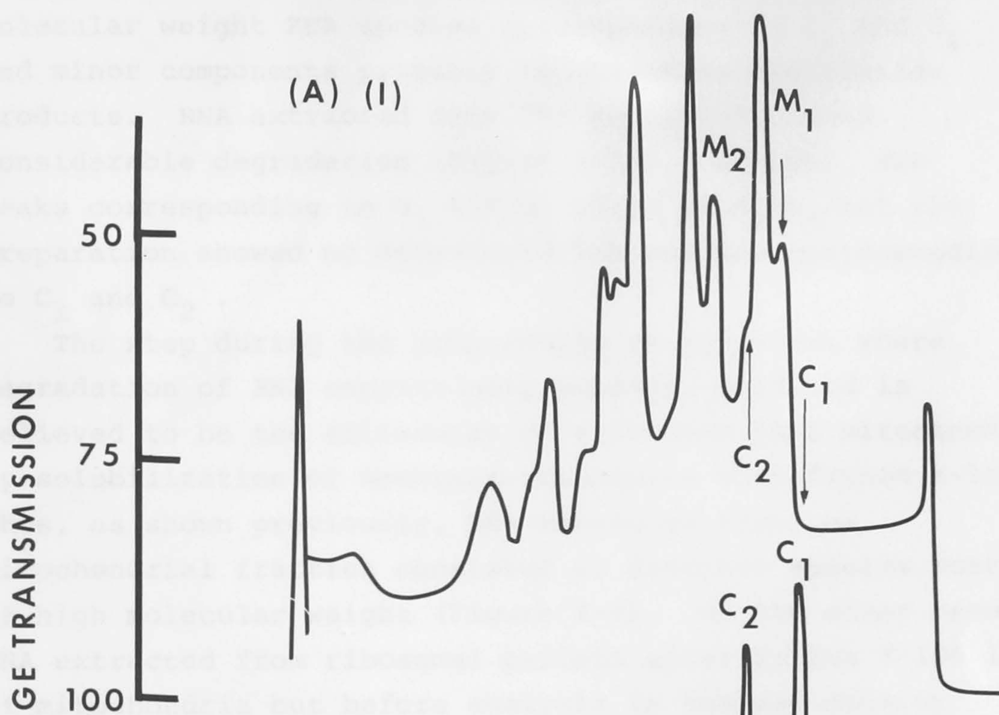
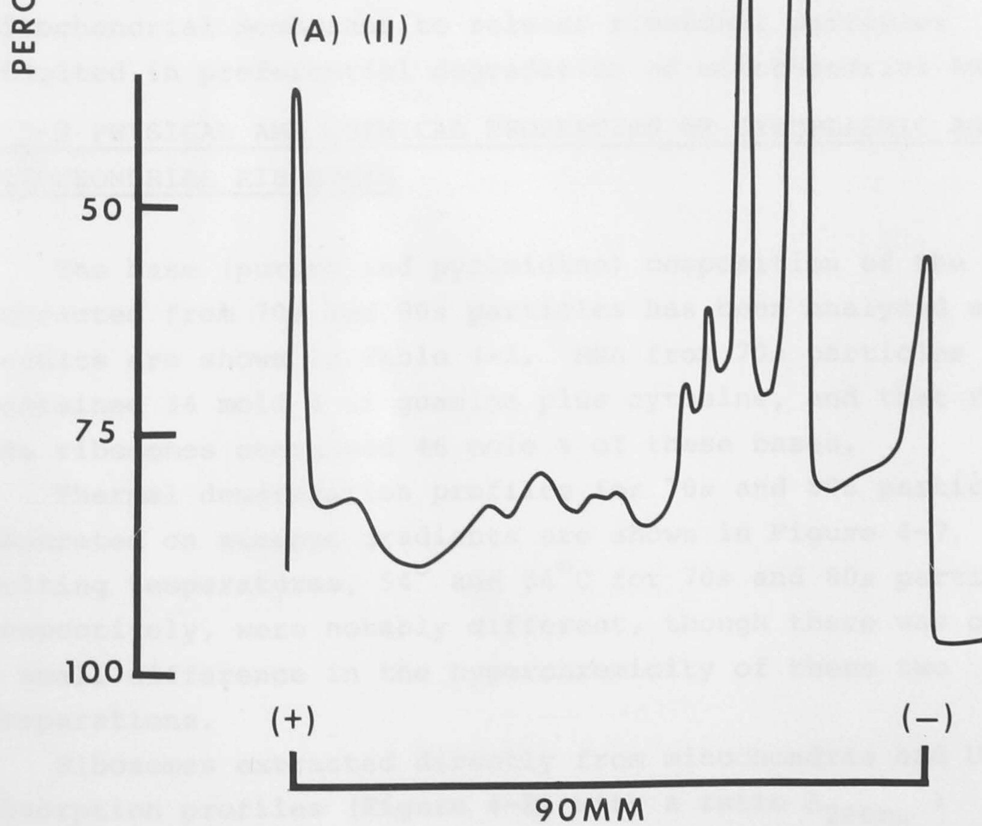

(A)
M1
C1
D
M2
C2
50
75
100
PERCENTAGE TRANSMISSION
(B)
50
75
100
(C)
50
75
100
(+)
(−)
90 MM

Figure 4-6(A). Electrophoretic patterns of RNA extracted from 70*s* and 80*s* particles.
70*s* and 80*s* particles were prepared on gradients by centrifugation of a ribosomal preparation of mitochondria. These particles were then separated and precipitated with ethanol overnight. Nucleic acids were extracted from ethanol precipitates with phenol-SDS as described in Section 4-2-7. The RNA extracts were then analyzed by electrophoresis in 2.4% polyacrylamide gels for 2 hr at 22°C. (A)(I) and (A)(II) are electrophoretic patterns of RNA extracted from 70*s* and 80*s* particles respectively.



(A) (I)
PERCENTAGE TRANSMISSION
50
75
100
M_1
M_2
C_1
C_2
C_1
C_2



(A) (II)
50
75
100
(+)
(–)
90MM

RNA from 80*s* particles showed (Figure 4-6a) two high molecular weight RNA species corresponding to C_1 and C_2 and minor components probably representing degradation products. RNA extracted from 70*s* particles showed considerable degradation (Figure 4-6a). However, RNA peaks corresponding to M_1 and M_2 could be seen, but the preparation showed no detectable RNA species corresponding to C_1 and C_2 .

The step during the preparation of ribosomes where degradation of RNA constituents probably occurred is believed to be the extraction of ribosomes from mitochondria by solubilization of membrane structures with Triton X-100. Thus, as shown previously, RNA extracted from the mitochondrial fraction consisted of discrete species mostly of high molecular weight (Figure 4-4). On the other hand RNA extracted from ribosomal pellets after Triton X-100 lysis of mitochondria but before analysis in sucrose density gradients was already degraded (Figure 4-6b). It was therefore concluded that lysis and solubilization of mitochondrial membranes to release ribosomal particles resulted in preferential degradation of mitochondrial RNA.

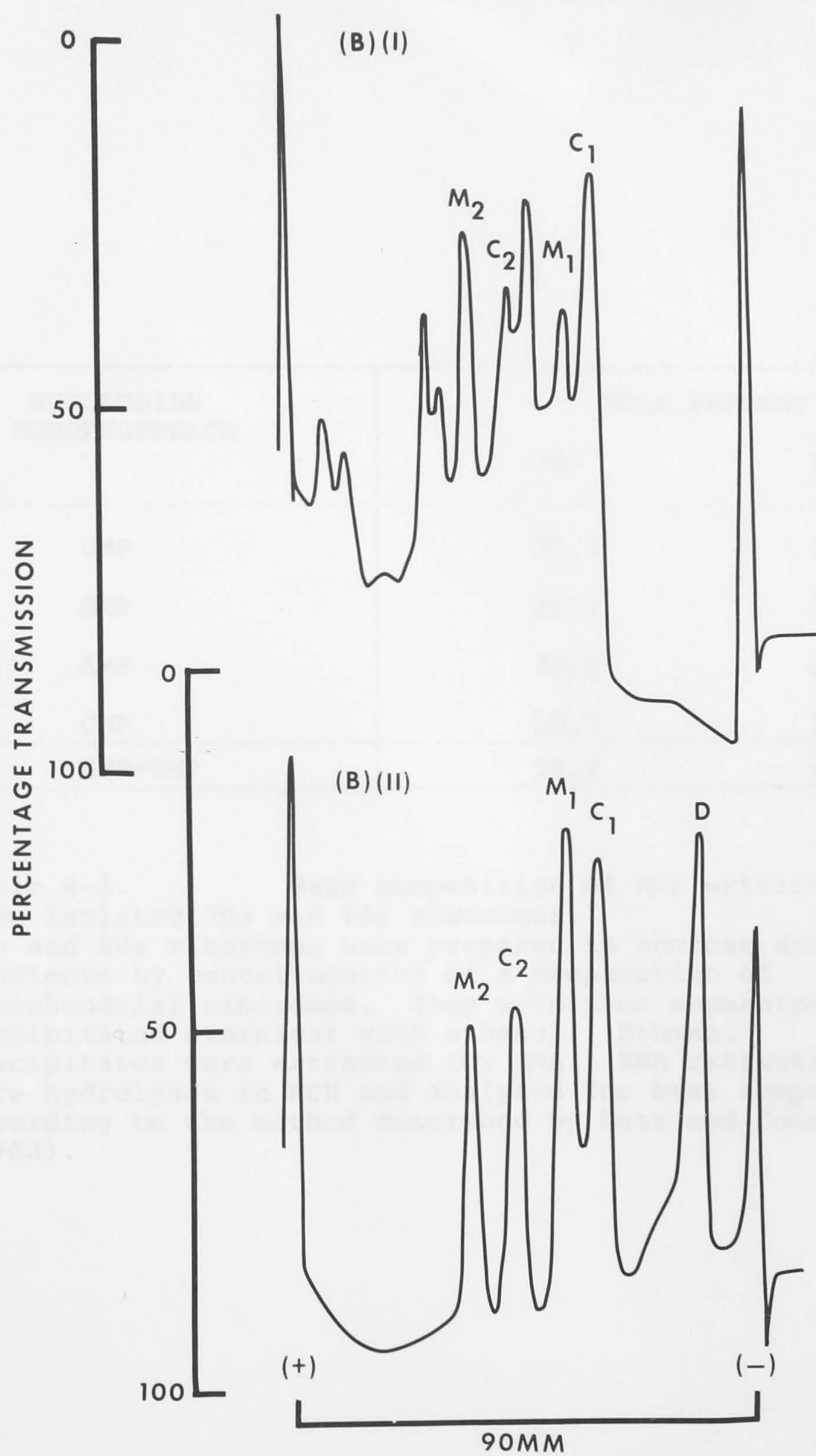
4-3-3 PHYSICAL AND CHEMICAL PROPERTIES OF CYTOPLASMIC AND MITOCHONDRIAL RIBOSOMES

The base (purine and pyrimidine) composition of the RNA extracted from 70*s* and 80*s* particles has been analyzed and results are shown in Table 4-1. RNA from 70*s* particles contained 34 mole % of guanine plus cytosine, and that from 80*s* ribosomes contained 46 mole % of these bases.

Thermal denaturation profiles for 70*s* and 80*s* particles separated on sucrose gradients are shown in Figure 4-7. The melting temperatures, 54° and 56°C for 70*s* and 80*s* particles respectively, were notably different, though there was only a small difference in the hyperchromicity of these two preparations.

Ribosomes extracted directly from mitochondria had UV absorption profiles (Figure 4-8) with a ratio $A_{260m\mu}$: $A_{235m\mu}$ of less than one, indicating gross contamination by protein. However, after purification on sucrose gradients,

Figure 4-6(B). Degradation of mitochondrial RNA consequent to the lysis and solubilization of mitochondrial membranes.
A purified mitochondrial preparation was divided into two portions. One portion was extracted with phenol-SDS for nucleic acids, the other was lyzed and solubilized with Triton X-100 to obtain ribosomal particles as described in Section 4-2-5. RNA was then extracted by phenol-SDS from the ribosomal particles. (B)(I) and (B)(II) are the electrophoretic patterns of nucleic acids of the ribosomal and mitochondrial pellets respectively.



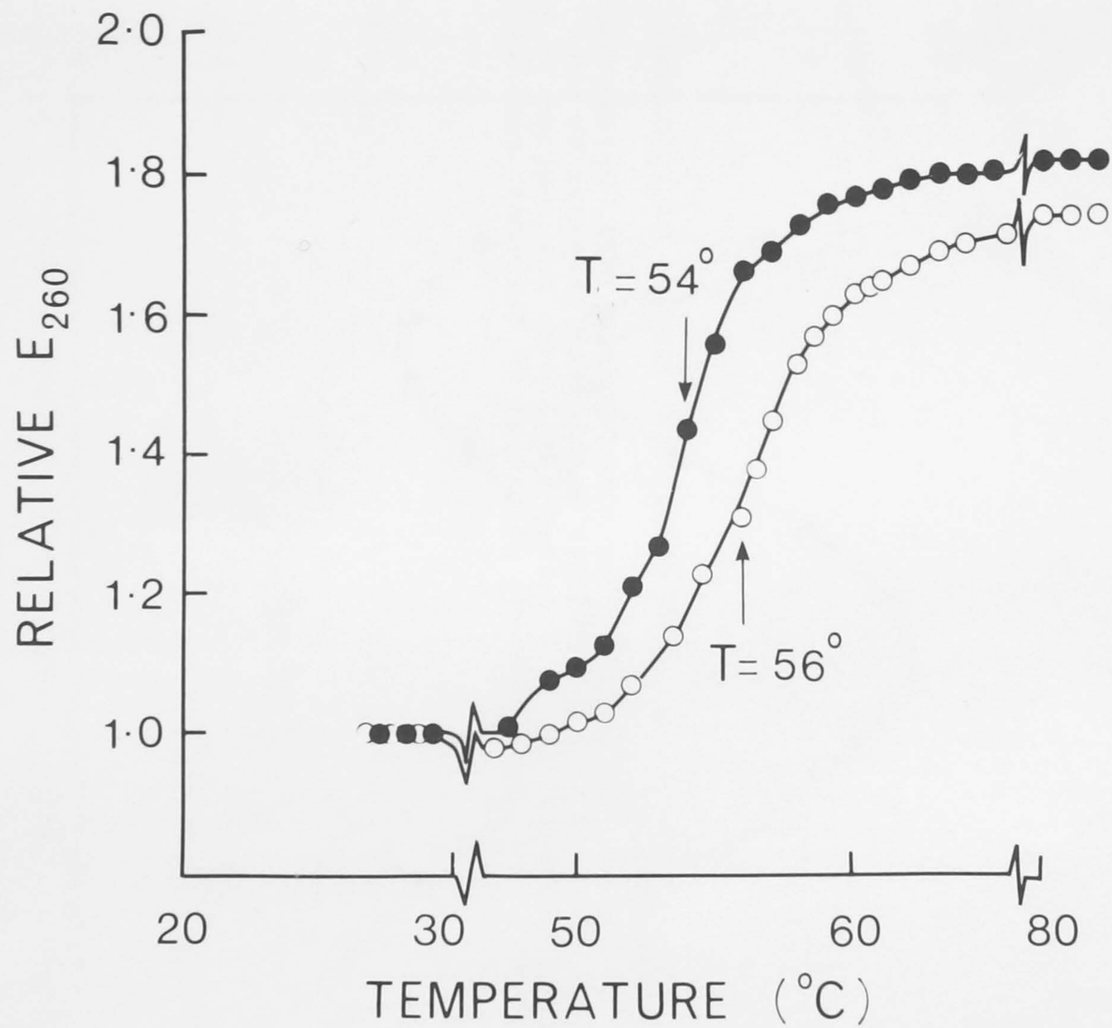
(I) RIBOSOMAL PELLET

(II) MITOCHONDRIAL PELLET

NUCLEOSIDE MONOPHOSPHATE	MOLE PERCENT	
	70*s*	80*s*
UMP	32.1	29.9
GMP	24.1	30.0
AMP	33.1	23.9
CMP	10.7	16.2
GMP+CMP	34.8	46.2

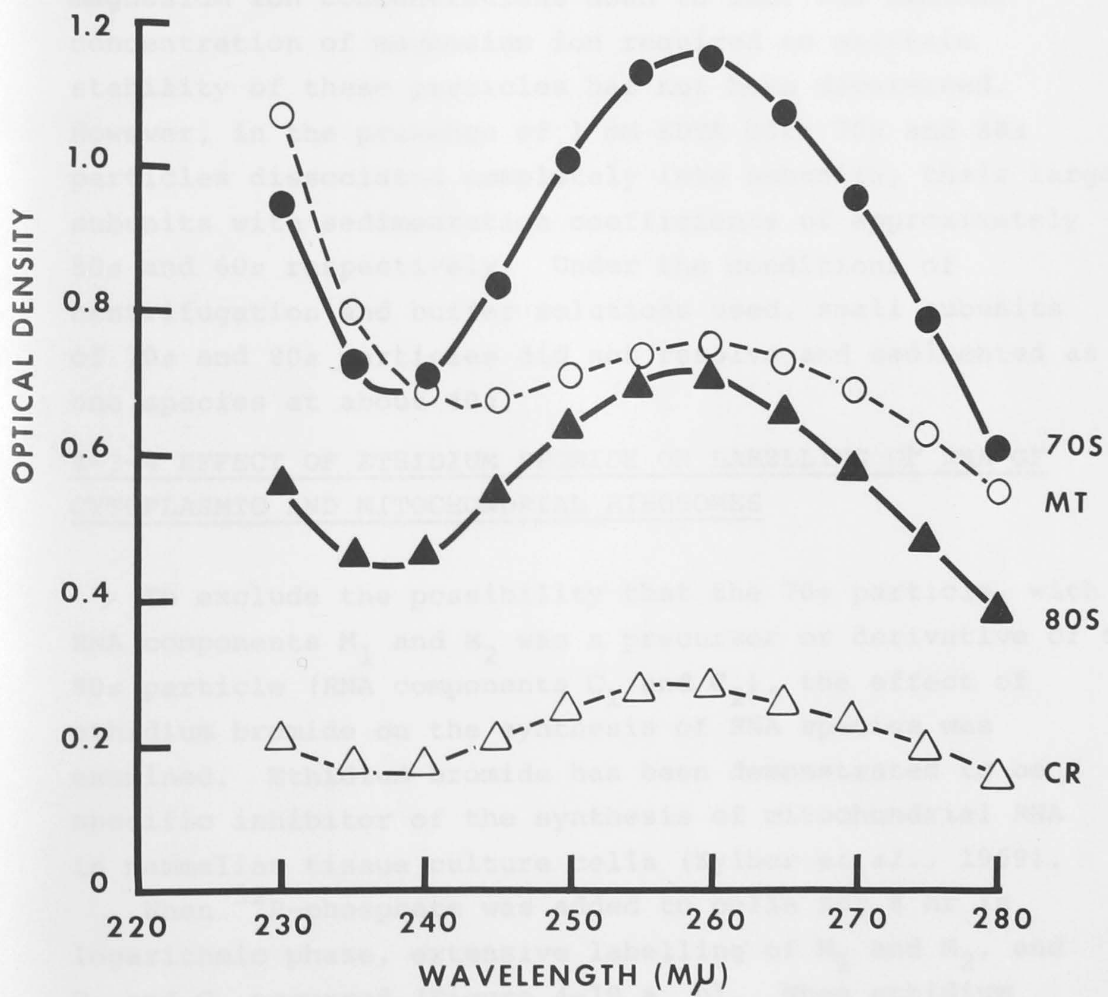
Table 4-1. Base composition of RNA extracted from isolated 70*s* and 80*s* ribosomes.
70*s* and 80*s* ribosomes were prepared in sucrose density gradients by centrifugation of a preparation of mitochondrial ribosomes. They were then separated and precipitated overnight with ethanol. Ethanol precipitates were extracted for RNA. RNA extracts were hydrolysed in KOH and analysed for base composition according to the method described by Katz and Coomb (1963).

Figure 4-7. Thermal denaturation of 70*s* and 80*s* particles.
70*s* and 80*s* particles were prepared on gradients by centrifugation of a ribosomal preparation of mitochondria. They were separated and appropriately diluted with AMT buffer (NH_4Cl 0.1M, $MgCl_2$ 0.01M, Tris HCl 0.01M pH 7.6) to give an $O.D._{260\ m\mu}$ of 0.2 - 0.3 at 27°C. Absorptions at 260 mμ of suspensions of these particles were recorded automatically as the temperature of the suspensions was increased. Experimental details are given in Section 4-2-11. The increase of UV absorption is referred to as hyperchromicity. The ordinate is relative absorption at 260 mμ (indicating $E_{260\ m\mu}$ at any temperature relative to $E_{260\ m\mu}$ at 27°C).



2·0
1·8
1·6
1·4
1·2
1·0
RELATIVE E_{260}
T = 54°
T= 56°
20
30
50
60
80
TEMPERATURE (°C)

Figure 4-8. UV absorption spectra of ribosomal preparations.
Ribosomal preparation from mitochondria (MT), cytoplasmic ribosomes (CR), 70*s* and 80*s* particles (prepared by the method described in Figure 4-6(A)) were resuspended in AMT buffer and their optical densities at different wavelengths determined.


OPTICAL DENSITY
1.2
1.0
0.8
0.6
0.4
0.2
0
220
230
240
250
260
270
280
WAVELENGTH (MU)
70S
MT
80S
CR

70*s* and 80*s* particles gave ratios of 1.7 and 1.9 for $A_{260m\mu}$: $A_{235m\mu}$ and $A_{260m\mu}$: $A_{280m\mu}$ respectively, indicating ribonucleoprotein particles with little extraneous protein contamination (Petermann, 1964). Maximum absorbance of particle suspensions of 70*s* and 80*s* was at 258 mμ and minimum at 235 mμ (Figure 4-8).

Effects of magnesium ions and of the chelating agent EDTA on the stability of 70*s* and 80*s* ribosomes are shown in Figure 4-9. 70*s* and 80*s* particles were stable in magnesium ion concentrations down to lmM; the minimal concentration of magnesium ion required to maintain stability of these particles has not been determined. However, in the presence of 1 mM EDTA both 70*s* and 80*s* particles dissociated completely into subunits, their large subunits with sedimentation coefficients of approximately 50*s* and 60*s* respectively. Under the conditions of centrifugation and buffer solutions used, small subunits of 70*s* and 80*s* particles did not resolve and sedimented as one species at about 40*s*.

4-3-4 EFFECT OF ETHIDIUM BROMIDE ON LABELLING OF RNA OF CYTOPLASMIC AND MITOCHONDRIAL RIBOSOMES

To exclude the possibility that the 70*s* particle, with RNA components M_1 and M_2 was a precursor or derivative of the 80*s* particle (RNA components C_1 and C_2), the effect of ethidium bromide on the synthesis of RNA species was examined. Ethidium bromide has been demonstrated to be a specific inhibitor of the synthesis of mitochondrial RNA in mammalian tissue culture cells (Zylber *et al.*, 1969).

When ^{32}P-phosphate was added to cells for 6 hr in logarithmic phase, extensive labelling of M_1 and M_2, and C_1 and C_2 occurred (Figure 4-10 a, b). When ethidium bromide was added at the same time as the isotope there was no inhibition of labelling of C_1 and C_2, from cytoplasmic ribosomes, nor of these two species associated with mitochondria (Figure 4-10 c, d). There was also no apparent effect of ethidium bromide on growth or the synthesis of cytochrome *c* over this period. By contrast, there was

Figure 4-9. Dissociation into subunits of ribosomal particles.

Stability of ribosomal particles was examined by sedimentation in sucrose density gradients (10-30% (w/v) in NH_4Cl 0.1M and Tris HCl 0.01M pH 7.6) containing different concentrations of magnesium ion. 70*s* and 80*s* particles of ribosomal preparations of mitochondria were stable in 1mM Mg^{++} (A). Identical sedimentation patterns to that shown in (A) were obtained when the gradient solutions contained 2, 5 and 10 mM Mg^{++}. (B) shows the sedimentation pattern when the ribosomal preparation of mitochondria was sedimented through sucrose gradients containing 1 mM EDTA. (C) shows the sedimentation pattern when a cytoplasmic ribosomal preparation was sedimented through sucrose gradients containing 1 mM EDTA. Conditions of centrifugation were the same as described in Figure 4-1.

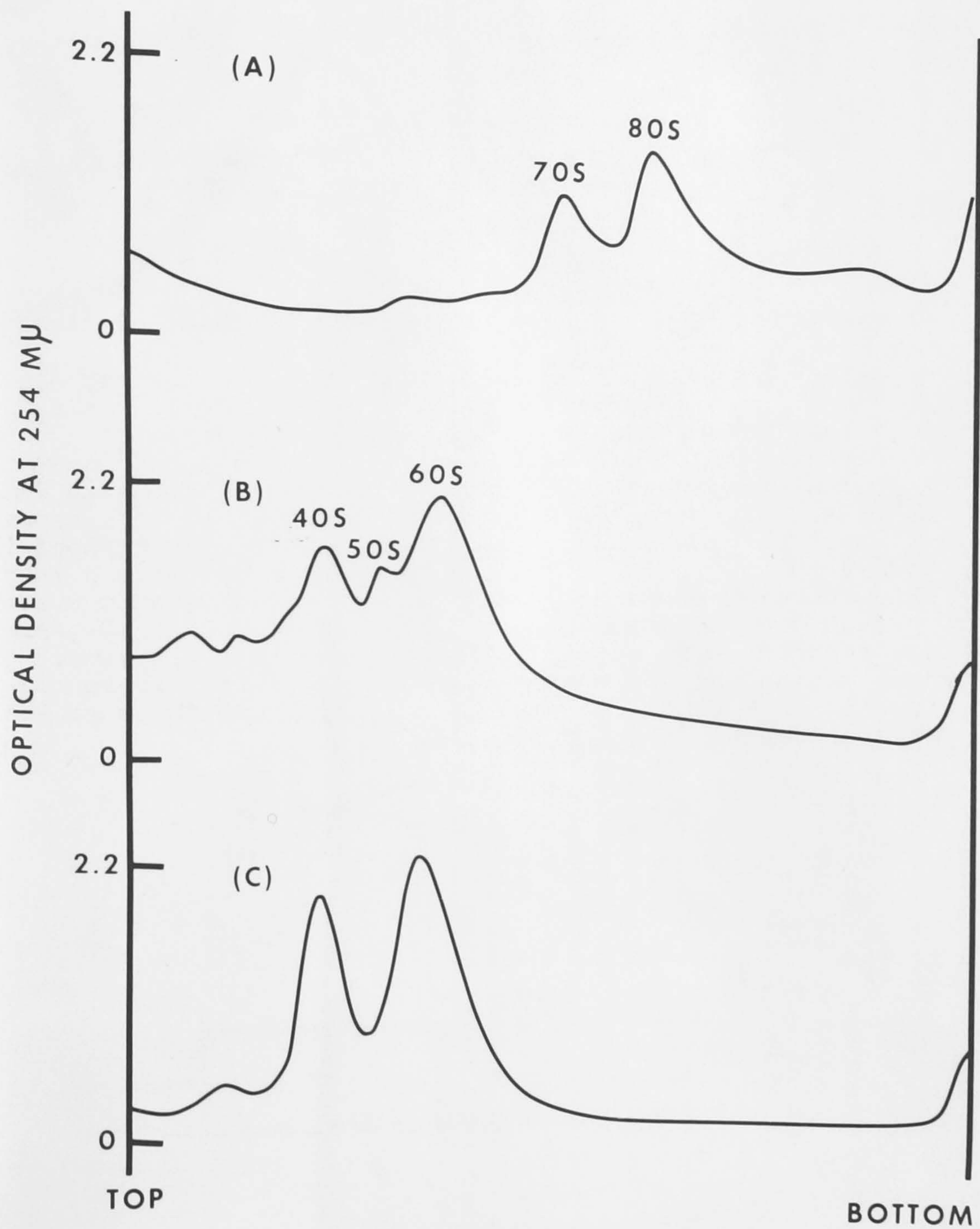
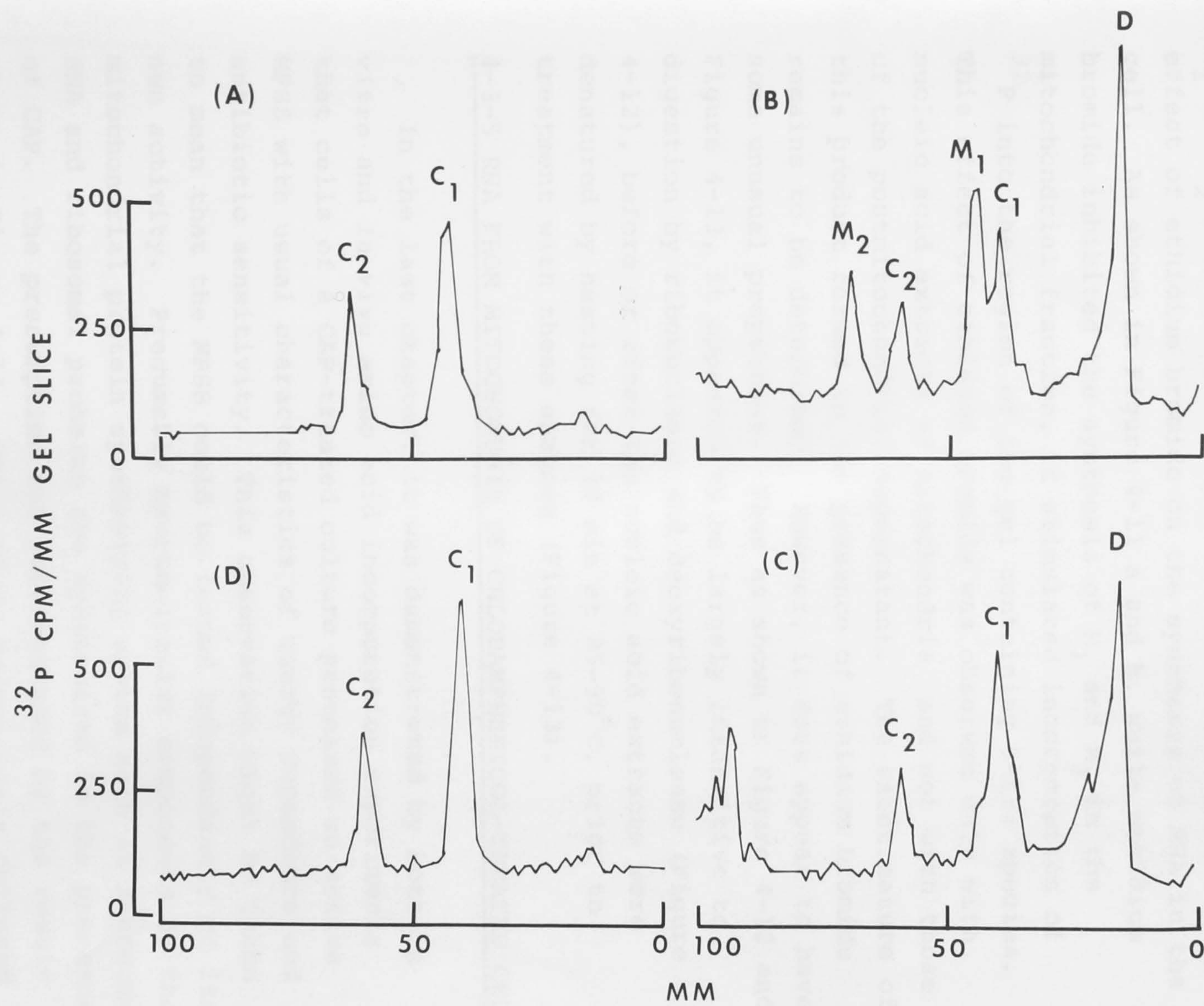

OPTICAL DENSITY AT 254 Mμ
2.2
0
(A)
70S
80S
2.2
0
(B)
40S
50S
60S
2.2
0
(C)
TOP
BOTTOM

Figure 4-10. Effect of ethidium bromide (EB) on the synthesis of RNA. A normal culture of *C. parapsilosis* grown in 2.5% glucose *Saccharomyces* medium was divided into 2 portions at time T=10 hr. To one portion EB (100 μg/ml) was added and the culture was allowed to equilibrate with EB for 5 min. 5 mCi of carrier-free ^{32}P-orthophosphate (sodium salt, pH 7, 181 Ci/mg P) per litre of culture were then added to both the control and EB-treated cultures. These cultures were incubated for 6 hr in the presence of ^{32}P. At the end of incorporation, cells were rapidly cooled and fractionated to obtain subcellular fractions as described in Section 4-2-2. Nucleic acids were then extracted from subcellular fractions and analyzed by electrophoresis in polyacrylamide gels as described in Sections 4-2-7 and 4-2-8. Gels were then frozen, sliced and hydrolyzed for determination of distribution of radio-activity as described in Section 4-2-9. Electrophoresis was carried out at 22°C for 4 hr. (A) and (D) are ^{32}P-labelling of cytoplasmic ribosomal RNA of the normal and EB-treated cells respectively. (B) and (C) are ^{32}P-labelling of nucleic acids extracted from mitochondria of the normal and EB-treated cell. Gels were loaded with 25 μg of cytoplasmic rRNA containing 10000 cpm or 50 μg of nucleic acids of mitochondria containing 20000 cpm.


(A)
(B)
(C)
(D)
C1
C2
M1
M2
D
500
250
0
100
50
0
32P CPM/MM GEL SLICE
MM

complete inhibition of labelling of M_1 and M_2 (Figure 4-10 c) in mitochondria from these cells.

However, it was observed that specific inhibition of M_1 and M_2 of the mitochondrial fraction was not the only effect of ethidium bromide on the synthesis of RNA in the cell. As shown in Figure 4-11 a and b, while ethidium bromide inhibited the synthesis of M_1 and M_2 in the mitochondrial fraction, it stimulated incorporation of ^{32}P into the region of the gel containing 9-12*s* species. This effect of ethidium bromide was observed only with nucleic acid extracts of mitochondria and not with those of the postmitochondrial supernatant. The exact nature of this product formed in the presence of ethidium bromide remains to be determined. However, it does appear to have some unusual properties. Thus as shown in Figure 4-12 and Figure 4-13, it appeared to be largely insensitive to digestion by ribonuclease and deoxyribonuclease (Figure 4-12), before or after the nucleic acid extracts were denatured by heating for 10 min at 85-90°C, prior to treatment with these enzymes (Figure 4-13).

4-3-5 RNA FROM MITOCHONDRIA OF CHLORAMPHENICOL-TREATED CELLS

In the last chapter, it was demonstrated by both *in vitro* and *in vivo* amino acid incorporation experiments that cells of a CAP-treated culture possessed an active MPSS with usual characteristics of energy dependence and antibiotic sensitivity. This observation might be taken to mean that the MPSS could be formed independently of its own activity. Presumably macromolecular components of the mitochondrial protein synthesising system such as ribosomal RNA and ribosomal proteins are synthesised in the presence of CAP. The presumption was substantiated by the result shown in Figure 4-14. Cells of *C. parapsilosis* cultured in the presence of CAP synthesised high molecular weight mitochondrial RNA species M_1 and M_2. Similar results have been reported by Davey, Yu and Linnane (1969) in *S. cerevisiae*.

Figure 4-11. Stimulation of ^{32}P-incorporation by EB into 9-12*s* material of nucleic acid extracts of mitochondria.
Incorporation of ^{32}P into nucleic acids was carried out as described in Figure 4-10. Nucleic acids extracted from mitochondria were analyzed by electrophoresis in polyacrylamide gels for 2 hr. (A) and (B) indicate ^{32}P-labelling of nucleic acid extracts of mitochondria of the normal and EB-treated cells respectively. 9-12*s* material was identified by the heterogenous labelling of the region between slice number 45 and 70. Gels were loaded with 50 μg of nucleic acids containing 15000 cpm (A) and 20000 cpm (B).

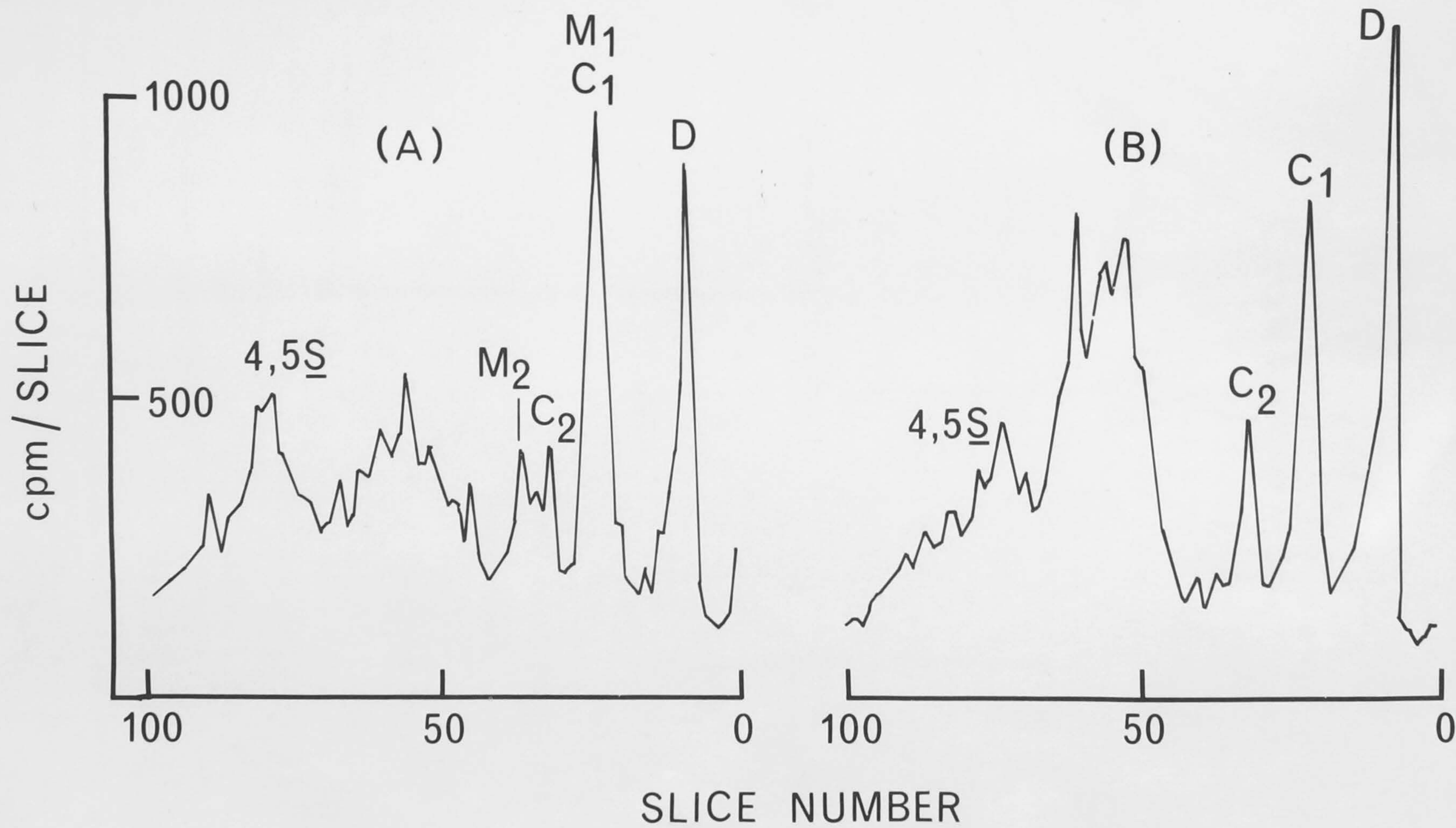
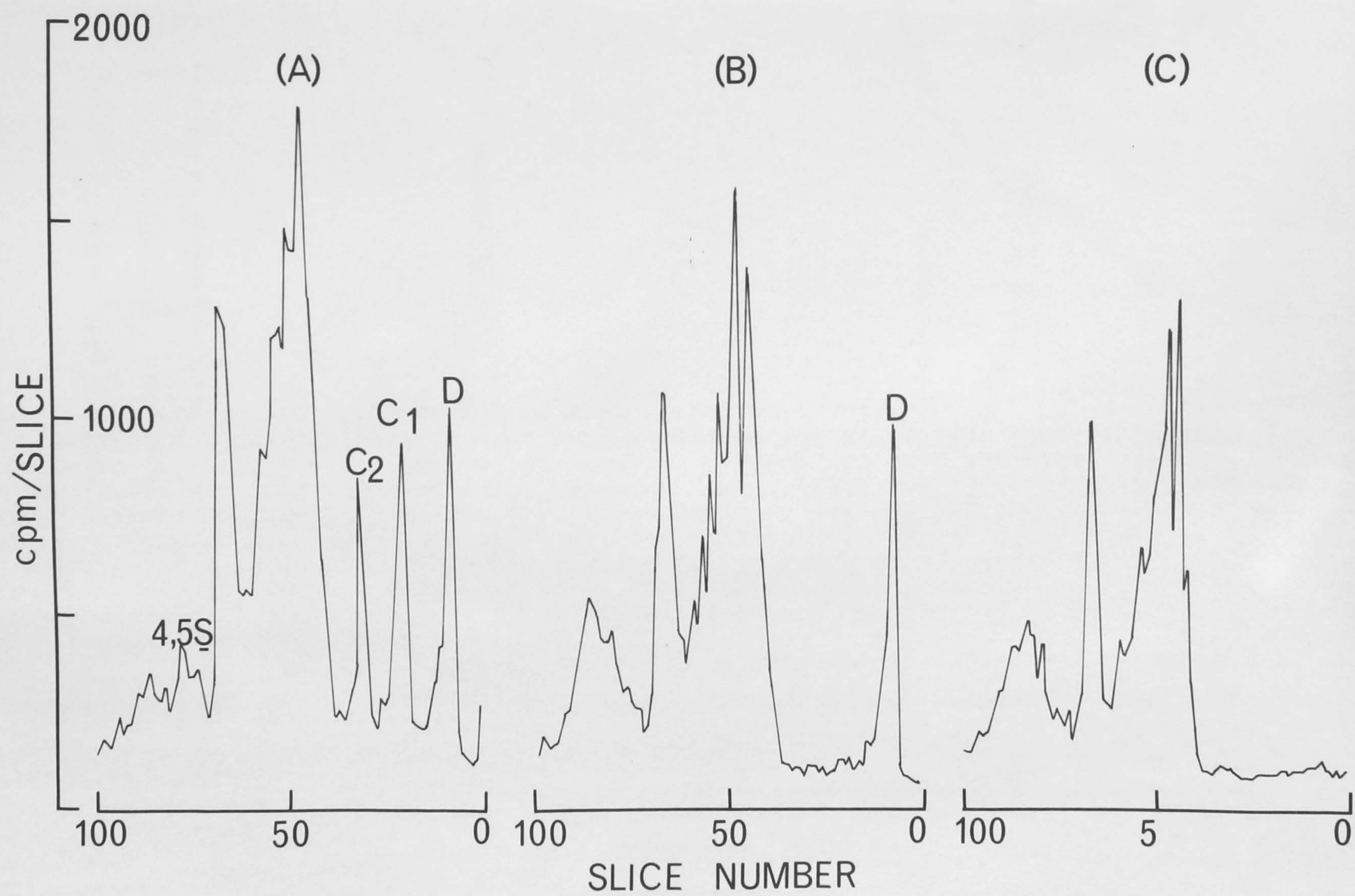
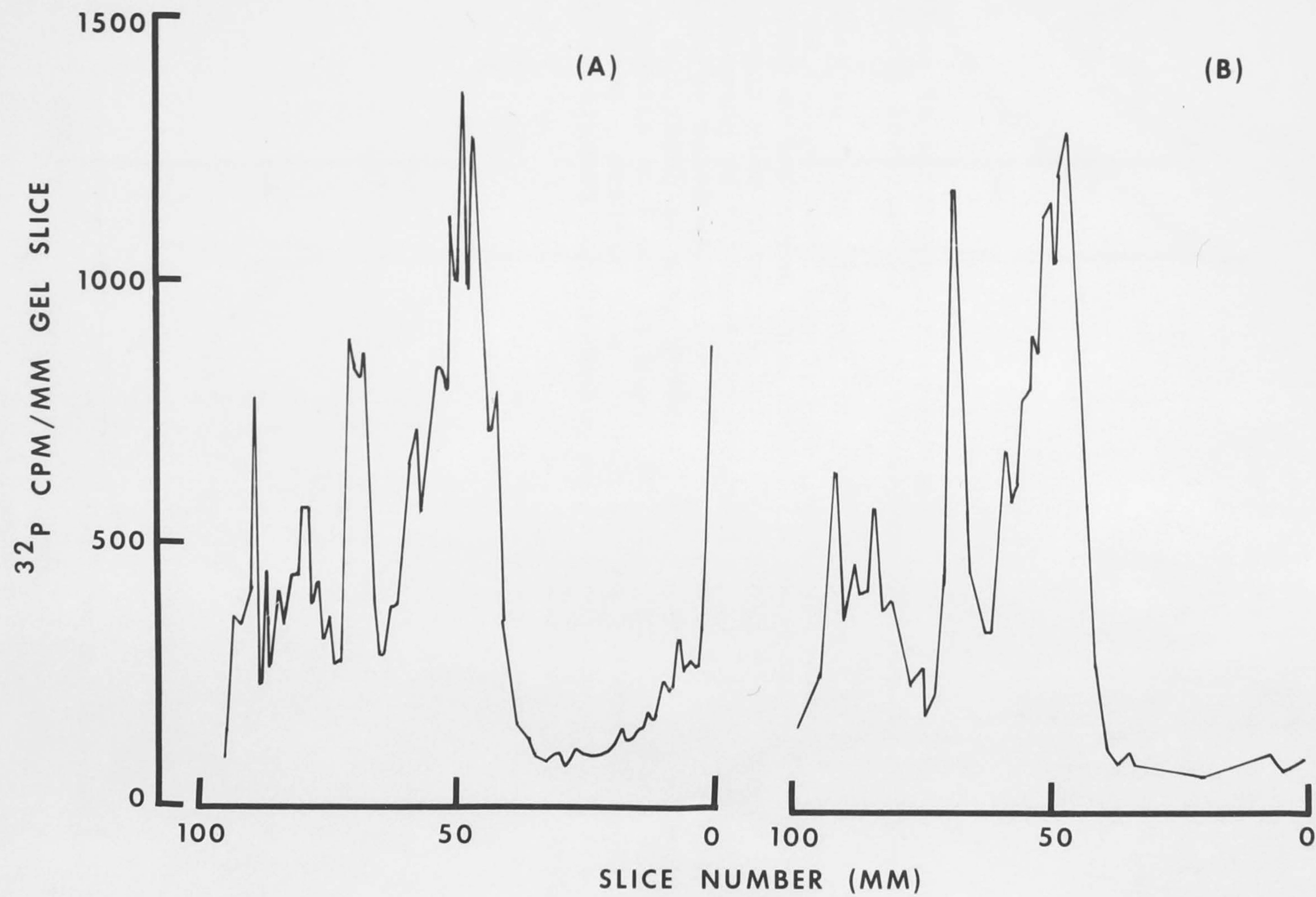

cpm / SLICE
1000
500
(A)
M1
C1
D
4,5S
M2
C2
100
50
0
(B)
D
C1
C2
4,5S
100
50
0
SLICE NUMBER

Figure 4-12. Effect of digestion by RNase and DNase on the EB-stimulated ^{32}P-incorporation into the 9-12*s* material.
Incorporation of ^{32}P by whole cells was carried out as described in Figure 4-10 except that 10 mCi of carrier-free ^{32}P-orthophosphate (181 Ci/mg P, sodium salt, pH 7) was added per litre of EB-treated (100 μg/ml) culture. Nucleic acid extracts of mitochondria were dissolved in electrophoresis buffer (Tris acetate 0.04M, sodium acetate 0.02M pH 7.8, sodium EDTA 0.001M) and then incubated with RNase (50 μg/ml) at 37°C for 5 min. An aliquot of the reaction mixture was then analyzed by electrophoresis in a 2.4% polyacrylamide gel. Another aliquot of the RNase-treated mixture was adjusted to 30 mM magnesium sulphate and 10 μg/ml DNase, and this mixture was incubated further for 5 min at 37°C. An aliquot of this reaction mixture was also analyzed by electrophoresis. (A), control not treated with nucleases; (B), treated with RNase; (C), treated with RNase and DNase. Gels were loaded with 50 μg of mitochondrial nucleic acids containing 30000 cpm.



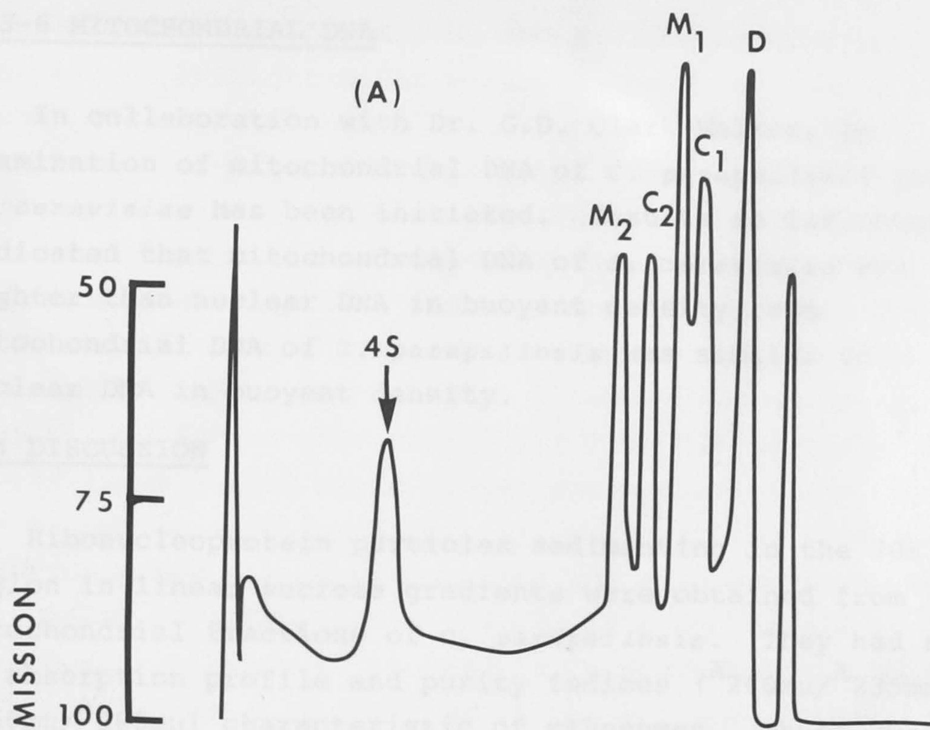
2000
1000
cpm/SLICE
(A)
(B)
(C)
4,5S
C_2
C_1
D
D
100
50
0
100
50
0
100
5
0
SLICE NUMBER

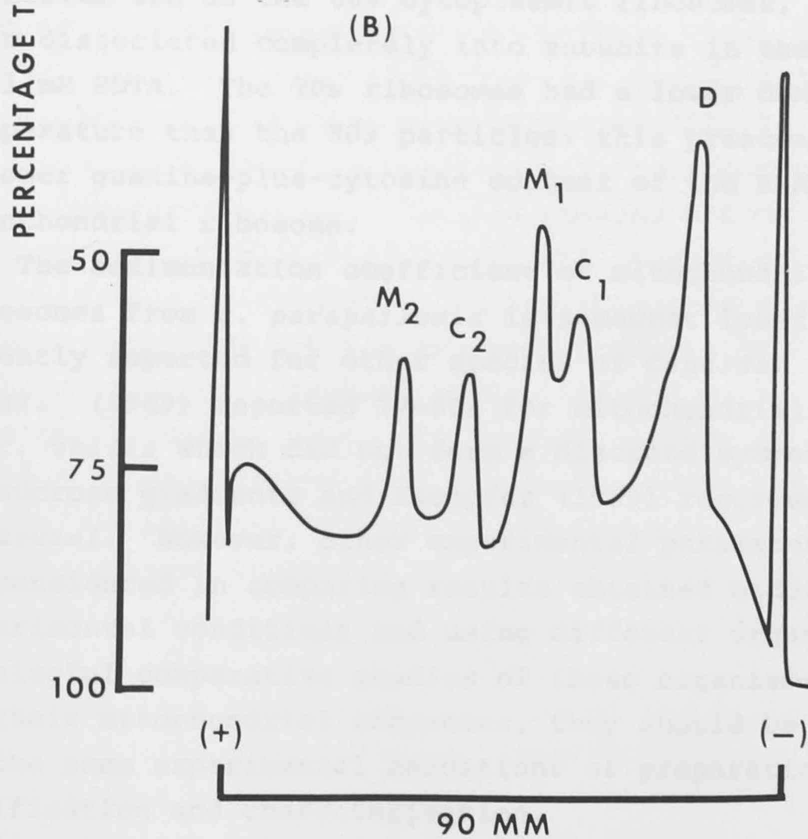
Figure 4-13. Effect of thermal denaturation and nuclease digestion on the EB-stimulated ^{32}P-labelling.
^{32}P-incorporation by whole cells in the presence of EB was carried out as described in Figure 4-12. The nucleic acid extract of the mitochondrial fraction was dissolved in electrophoresis buffer and heated at 85-90°C for 10 min. It was then rapidly cooled to 37°C. Digestion with RNase (at 50 µg/ml) was carried out. An aliquot of the reaction mixture was analyzed by gel-electrophoresis. A further aliquot of the RNase-treated mixture was then adjusted to 30 mM magnesium sulphate and 10 µg/ml DNase, and incubated at 37°C for 5 min. The latter reaction mixture was then analyzed. (A), heat-denatured and RNase digested; (B), heat-denatured and RNase plus DNase digested. Loadings were similar to those in Figure 4-12.



1500
1000
500
0
(A)
(B)
^{32}P CPM/MM GEL SLICE
100
50
0
100
50
0
SLICE NUMBER (MM)

Figure 4-14. Electrophoretic patterns of nucleic acids extracted from mitochondria of CAP-treated cells of *C. parapsilosis*.
Cells of a CAP-treated (3 mg/ml) culture of *C. parapsilosis* at T=24 hr were harvested, washed and fractionated to obtain a purified mitochondrial fraction as described in Section 4-2-2 and 4-2-3 (Method C). Nucleic acids were then extracted from the mitochondrial fraction by phenol-SDS (Section 4-2-7) and analyzed by electrophoresis at room temperature in 2.4% polyacrylamide gels as described in Section 4-2-8. (A) and (B) are 2 hr and 4 hr electrophoretic patterns of the nucleic acid extract. Loading of gels was routinely 50 μg of mitochondrial nucleic acids per gel.


(A)
M_1
D
C_1
C_2
M_2
4S
50
75
100
PERCENTAGE TRANSMISSION


(B)
D
M_1
C_1
M_2
C_2
50
75
100
(+)
(−)
90 MM

4-3-6 MITOCHONDRIAL DNA

In collaboration with Dr. G.D. Clark-Walker, an examination of mitochondrial DNA of *C. parapsilosis* and *S. cerevisiae* has been initiated. Results so far obtained indicated that mitochondrial DNA of *S. cerevisiae* was lighter than nuclear DNA in buoyant density, and mitochondrial DNA of *C. parapsilosis* was similar to nuclear DNA in buoyant density.

4-4 DISCUSSION

Ribonucleoprotein particles sedimenting in the 70*s* region in linear sucrose gradients were obtained from mitochondrial fractions of *C. parapsilosis*. They had a UV absorption profile and purity indices ($^{A}260m\mu/^{A}235m\mu$; $^{A}260m\mu/^{A}280m\mu$) characteristic of ribosomes. These 70*s* particles were as stable in low concentrations of magnesium ion as the 80*s* cytoplasmic ribosomes, and like them dissociated completely into subunits in the presence of 1 mM EDTA. The 70*s* ribosomes had a lower denaturation temperature than the 80*s* particles; this presumably reflects a lower guanine-plus-cytosine content of the RNA of the mitochondrial ribosome.

The sedimentation coefficient of mitochondrial ribosomes from *C. parapsilosis* is somewhat lower than those recently reported for other species of *Candida*. Vignais *et al.* (1969) reported 77-80*s* for mitochondrial ribosomes of *C. utilis* which did not form a discrete symmetrical peak in sucrose gradients and Kaempfer (1969) reported 76*s* for *C. krusei*. However, other experimental parameters have to be considered in comparing results obtained under different experimental conditions and using different organisms. For meaningful comparative studies of these organisms in terms of their mitochondrial ribosomes, they should be subjected to the same experimental conditions of preparation, purification and characterization.

The bulk of the RNA extracted directly from mitochondrial fractions can be accounted for by high molecular weight

species designated C_1, M_1, C_2 and M_2 (the evaluation of recovery of RNA containing material showed that over 80% of the material solubilized by Triton X-100 was localised in the ribosomal pellet). The concomitant enrichment of 70*s* particles in ribosomal preparations of mitochondria and of high molecular weight RNA species M_1 and M_2, together with results of mixing experiments indicate that M_1 and M_2 are RNA components of the 70*s* ribosome. Experiments aimed at a direct demonstration that M_1 and M_2 are ribosomal RNA of the 70*s* particle proved inconclusive since direct extraction of RNA from 70*s* particles resulted in partial degradation. The RNA extracted from 80*s* ribosomes from the same gradient (which presumably came from contamination of mitochondria by cytoplasmic ribosomes) showed little degradation. This may be taken as an indication of the greater instability of the RNA of 70*s* particles or mitochondrial RNA. Attempts to prevent this degradation, using various combinations of high pH, dithiothreitol and other ribonuclease inhibitors during lysis of mitochondria have not been successful.

In terms of the base composition of RNA, 70*s* and 80*s* particles were found to be different, suggesting that they are of separate origin. The low guanine and cytosine content of high molecular weight RNA from mitochondria has generally been interpreted implicitly as evidence that mitochondrial RNA species are gene products of mitochondrial DNA (Fauman, Rabinowitz and Getz, 1969). Data on ^{32}P labelling of RNA in the presence of ethidium bromide showed clearly that the synthesis of RNA components of the 70*s* particle, M_1 and M_2, is inhibited by ethidium bromide whereas synthesis of C_1 and C_2 of 80*s* particles is not. This result indicates that the 70*s* particle is not an artifact derived from 80*s* particles. Nor, for the same reason, is it a precursor. In view of the reported specific inhibitory effect of ethidium bromide on transcription of mitochondrial genome (Zylber *et al.*, 1969), it is tentatively concluded that M_1 and M_2 are transcriptional products of the mitochondrial genome.

Synthesis of low molecular weight RNA (4*s* and 5*s*) has not been looked at rigorously. It has been reported by Lizardi and Luck (1971) that mitochondrial ribosomes of *Neurospora crassa,* unlike cytoplasmic ribosomes, do not contain 5*s* RNA. If this can be extended to *C. parapsilosis*, synthesis of the 5*s* RNA present in extracts of mitochondrial fractions as a constituent of contaminating cytoplasmic ribosomes would be expected to be insensitive to inhibition by ethidium bromide like that of C_1 and C_2. On the other hand, with mammalian tissues both inhibitor studies and hybridization experiments suggest that mitochondrial-specific tRNA (4*s* RNA) is a transcriptional product of mitochondrial DNA (see review by Kuntzel, 1971). Thus, mitochondrial-specific 4*s* RNA, like mitochondrial RNA M_1 and M_2, should be inhibited by ethidium bromide in *C. parapsilosis*. However, experimental data presented here do not permit the conclusion that synthesis of 4*s* RNA found in RNA extracted from mitochondrial fractions of *C. parapsilosis* is inhibited by ethidium bromide. This is firstly, because RNA extracts of mitochondrial fractions were contaminated by cytoplasmic ribosomes and hence by 4*s* RNA of extramitochondrial origin; secondly, because the polyacrylamide gels used in these experiments do not resolve RNA species distinctively in the low molecular weight region. Resolution is made more difficult by the fact that synthesis of materials in the 9-12*s* region of the gel is stimulated by ethidium bromide.

When ethidium bromide was used to inhibit synthesis of mitochondrial RNA (21*s*, 12*s* and 4*s*) in HeLa cells (Zylber *et al.,* 1969), other effects of the drug were not examined. As presented in this chapter, ethidium bromide in addition to inhibiting preferentially the synthesis of mitochondrial RNA, stimulated ^{32}P incorporation into 9-12*s* material of mitochondria. It was thought that this material may be RNA-DNA hybrids and/or double stranded RNA molecules. However, this appeared unlikely from data presented on nuclease digestion. After denaturing the nucleic acid extract by heating at 85-90°C in an attempt to effect a separation of strands, the material was still largely insensitive to nuclease digestion. It is also unlikely

that the 9-12*s* material is of protein or phospholipid nature because the experimental procedure involved excludes such a possibility. Thus the chemical nature of this ^{32}P-labelled material remains undetermined.

Selective inhibition of the synthesis of mitochondrial DNA by ethidium bromide has been reported by Goldring *et al.* (1970) in *S. cerevisiae.* These authors show that in the presence of EB, pre-existing mitochondrial DNA is progressively degraded because these molecules become more sensitive to nuclease attack due to the intercalation of ethidium bromide. In petites generated by prolonged treatment with EB, no mitochondrial DNA could be detected. Although extensive treatment with EB appears to eliminate mitochondrial DNA, synthesis of mitochondrial DNA of low molecular weight continued after brief EB-treatments. Petites isolated after EB-treatment for various short times contain mitochondrial DNA of reduced sizes which are in some cases as low as one-tenth of the normal (Goldring *et al.*, 1971). In view of the nonmutagenic nature of the inhibitory effect of EB and the similarity of buoyant density between mitochondrial and nuclear DNA of *C. parapsilosis,* it would therefore be extremely interesting to see if EB treatment will produce mitochondrial DNA of progressively diminishing sizes in the manner described by Goldring *et al.* (1970 b). Further comparative studies of mitochondrial DNA of *C. parapsilosis* and *S. cerevisiae* and their interaction with mutagens such as EB will throw light on the fundamental question of why obligate aerobes like *C. parapsilosis* are so-called petite-negative organisms.

CHAPTER 5

A COMPARATIVE STUDY OF THE MACROMOLECULAR COMPONENTS OF THE MPSS OF *C. PARAPSILOSIS* AND *S. CEREVISIAE* (II) MITOCHONDRIAL RIBOSOMES AND RNA OF *S. CEREVISIAE*.

CHAPTER 5

5-1 INTRODUCTION

In this chapter I shall describe experimental results obtained from *Saccharomyces cerevisiae* using techniques which had already proved successful for *Candida parapsilosis*. It would seem to be of great advantage to a comparative study of mitochondrial differentiation that experimental conditions and techniques are equivalent in as many respects as possible. Therefore, the procedures used in experiments described in this chapter were identical to those used for *C. parapsilosis* in the last chapter unless specified otherwise.

5-2 EXPERIMENTAL

5-2-1 GLUTARALDEHYDE FIXATION OF CELLS

Cells were collected by centrifugation from cultures, washed twice with large volumes of distilled water, resuspended at $0^{o}C$ in a volume of glutaraldehyde (5% v/v) equal to the volume of packed cells. The mixture was incubated with regular agitation at $0^{o}C$ for 30 min. Cells were then removed from the glutaraldehyde by centrifugation and washed twice with large volumes of cold distilled water. Glutaraldehyde-treated cells were again washed with sucrose-AMT buffer before disruption.

Disruption and fractionation of glutaraldehyde-treated cells was carried out in an identical fashion to that described in Section 4-2-2.

5-2-2 FUNCTIONAL CHARACTERIZATION OF RIBOSOMAL PARTICLES

Cells were collected from cultures by centrifugation, washed once with distilled water and once with glucose phosphate buffer (potassium phosphate 0.05M (pH 7.0), glucose 0.01% w/v). Washed cells were resuspended in glucose phosphate buffer at about 10 mg dry weight of cells per ml buffer, and divided into equal portions. Antibiotics were added to individual portions of cell

suspensions as specified. Cell suspensions were allowed to equilibrate with antibiotics for 15 min at 29°C with shaking. After equilibration, cells were pulsed-labelled for 10 min with ^3H-leucine (L-leucine-4-5-^3H, 100 μCi/ml, 34.1 Ci/mmole) at 50 μCi per 200 ml of cell suspension. After pulse-labelling, cell suspensions were immediately chilled by pouring onto ice, then centrifuged. Cells were then washed once with ice-cold distilled water, and once with sucrose-AMT buffer. Washed cells were then disrupted and fractionated according to procedure described under Section 4-2-2. Mitochondrial fractions were purified according to method A described in Section 4-2-3. Ribosomal particles were obtained from mitochondrial fractions and postmitochondrial supernatants according to procedures described in Sections 4-2-5 and 4-2-4 respectively.

Ribosomal preparations from mitochondria and postmitochondrial supernatants were analysed on sucrose density gradients. Ribosomal pellets resuspended in 1 ml of AMT buffer (containing about 2 mg of ribosomal material) were applied to 28 ml linear sucrose density gradients (10% - 20% w/v sucrose in AMT buffer). Gradients were centrifuged at 25000 rpm (Spinco, SW 25.1 rotor) for 4 hr at 4°C. The distribution of ribosomal particles in the gradient was measured at 254 mμ with an ISCO density gradient fractionator with a 2-mm light-path flow cell. 1 ml fractions of gradient material were collected into scintillation vials and radioactivities was determined by adding 10 ml of scintillation mixture (containing naphthalene 60 gm, PPO 4 gm, POPOP 0.2 gm, methanol 100 ml, ethylene glycol 20 ml, and dioxan to one litre).

5-2-3 CULTIVATION OF PETITE

A respiratory-deficient vegetative petite was derived from *Saccharomyces cerevisiae* strain M by ethidium bromide treatment. It was characterized by inability to grow on glycerol and had negligible respiratory activity. The absolute absorption spectrum of these cells showed only cytochrome *c* to be present. This organism was cultivated in *Saccharomyces* medium containing 2.5% glucose as described in Section 2-2-3.

5-2-4 CULTIVATION OF CELLS UNDER ANAEROBIC CONDITIONS

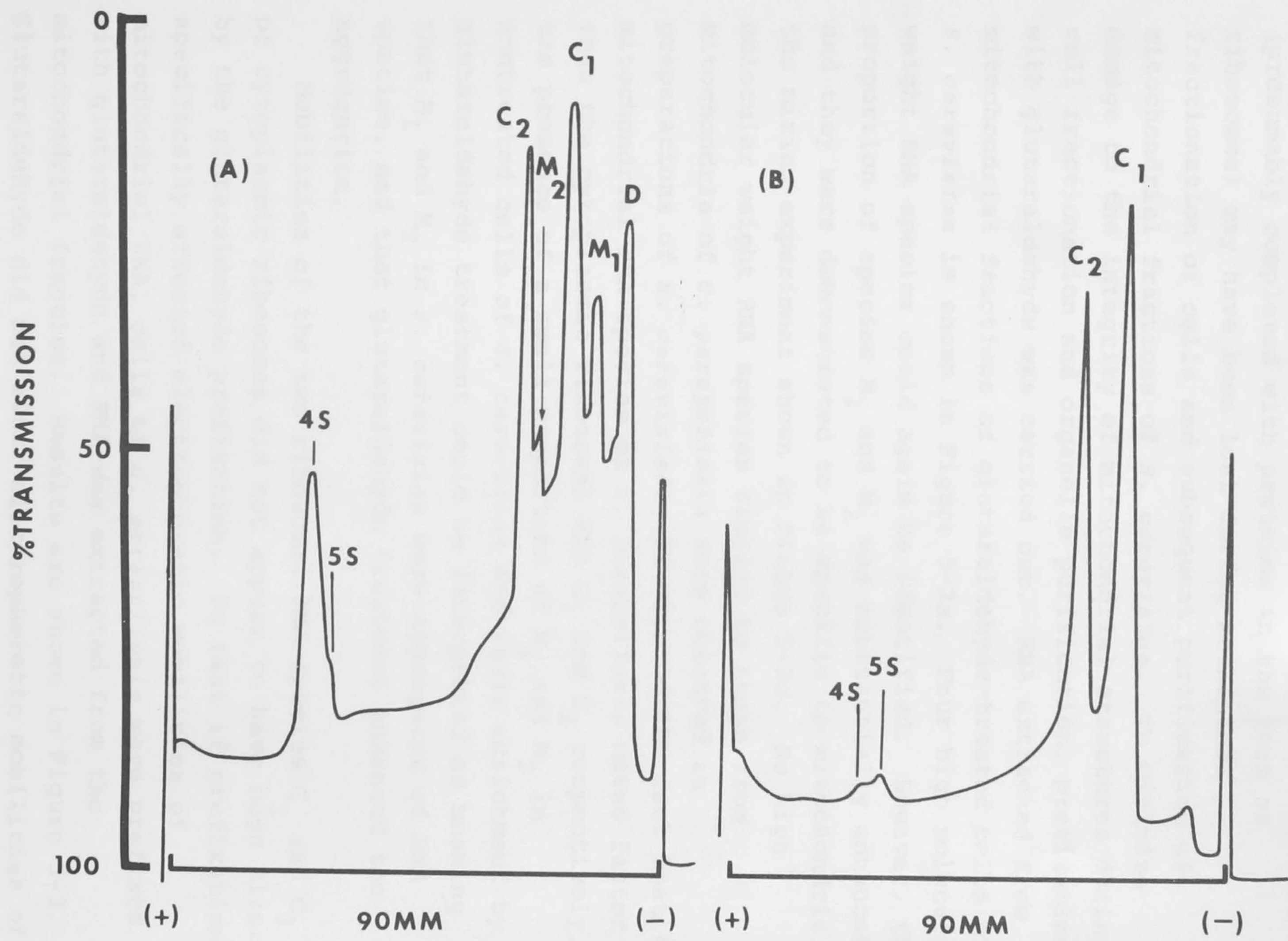
The liquid medium used for the cultivation of *S. cerevisiae* under anaerobic conditions was *Saccharomyces* medium plus Tween 80 (5 ml/l) and ergosterol (20 mg/l); the carbon source used was galactose 40 gm/l. Cells were grown at 29°C anaerobically in deep culture vessels (8 litres of culture in 10 litre-carboy) stirred magnetically. Cultures were flushed with oxygen-free nitrogen for 30 min prior to sealing the culture vessels with paraffin wax, and carbon dioxide generated during anaerobic fermentation was allowed to escape through a water trap as described by Jollow *et al.* (1968).

5-3 RESULTS

5-3-1 MITOCHONDRIAL RNA OF *S. CEREVISIAE*

Using the method described for the preparation and purification of mitochondria (method C of Section 4-2-4) from *C. parapsilosis*, RNA extracted from mitochondrial fractions of *S. cerevisiae* showed two major species and two minor species of high molecular weight RNA, as well as two low molecular weight species (Figure 5-1a). These were designated as M_1, C_1, M_2, C_2, 5*s* and 4*s* in order of increasing electrophoretic mobilities. These species have been identified to be RNA by their sensitivity towards digestion by ribonuclease and resistance to deoxyribonuclease. The high molecular weight species designated as D was identified to be DNA using similar criteria. The two predominant high molecular weight RNA species, C_1 and C_2 corresponded in electrophoretic mobility to the two ribosomal RNA species of cytoplasmic ribosomes respectively (Figure 5-1b). The two minor high molecular weight RNA species were considered to be of mitochondrial origin since they were not seen in cytoplasmic preparations. These two RNA species M_1 and M_2 appeared to be larger molecules than C_1 and C_2 respectively since they had lower mobility in gels. The relatively low yields of mitochondria-specific RNA in *S. cerevisiae* using methods which resulted in good yield of mitochondrial RNA in *C. parapsilosis* have been

Figure 5-1. Electrophoretic patterns of nucleic acid extract of mitochondria and RNA of cytoplasmic ribosomes of *S. cerevisiae*.
Mitochondria and cytoplasmic ribosomes were prepared as described in Section 4-2-3 (Method C) and Section 4-2-4 respectively. Nucleic acids were extracted from these subcellular fractions by phenol-SDS as described in Section 4-2-7 and analyzed by electrophoresis in 2.4% polyacrylamide gels at room temperature for 2 hr as described in Section 4-2-8. (A) and (B) are electrophoretic patterns of nucleic acid extract of mitochondria and RNA of cytoplasmic ribosomes respectively. Loading of gels was routinely 50 µg of mitochondrial nucleic acids or 25 µg of cytoplasmic rRNA.



(A)
(B)
%TRANSMISSION
0
50
100
4S
5S
C2
M2
C1
M1
D
(+)
90MM
(−)

attributed to possible differences between the rigidity or stability of mitochondrial membranes of *C. parapsilosis* and *S. cerevisiae*. High molecular weight RNA from mitochondria (presumably complexed with proteins in the form of ribosomes) may have been lost during mechanical fractionation of cells and subsequent purification of mitochondrial fractions of *S. cerevisiae*. To minimize damage to the integrity of mitochondrial structures during cell fractionation and organelle purification, prefixation with glutaraldehyde was carried out. RNA extracted from mitochondrial fractions of glutaraldehyde-treated cells of *S. cerevisiae* is shown in Figure 5-2a. Four high molecular weight RNA species could again be identified. However, the proportion of species M_1 and M_2 was substantially enhanced, and they were demonstrated to be specific to mitochondria by the mixing experiment shown in Figure 5-2c. No high molecular weight RNA species similar to those from mitochondria of *C. parapsilosis* were observed in preparations of *S. cerevisiae*. In view of the fact that the mitochondrial RNA species of *C. parapsilosis* moved faster than the cytoplasmic ribosomal RNA C_1 and C_2 respectively, the presence of a small proportion of M_1 and M_2 in nontreated cells of *S. cerevisiae* and their enrichment by glutaraldehyde treatment could be interpreted as meaning that M_1 and M_2 in *S. cerevisiae* were aggregates of RNA species, and that glutaraldehyde treatment enhanced the aggregation.

Mobilities of the two ribosomal RNA species C_1 and C_2 of cytoplasmic ribosomes did not appear to have been altered by the glutaraldehyde prefixation. To test if prefixation specifically affected electrophoretic mobilities of mitochondrial RNA, cells of *C. parapsilosis* were prefixed with glutaraldehyde and RNA was extracted from the mitochondrial fraction. Results are shown in Figure 5-3. Glutaraldehyde did not alter electrophoretic mobilities of cytoplasmic ribosomal RNA (Figure 5-3b) and did not affect mobilities of mitochondrial ribosomal RNA species from *C. parapsilosis* (Figure 5-3a). It appeared therefore that

Figure 5-2. Electrophoretic patterns of nucleic acid extract of mitochondria and RNA of cytoplasmic ribosomes of *S. cerevisiae* after prefixation of cells with glutaraldehyde. Cells of a culture of *S. cerevisiae* grown in 1.0% glucose *Saccharomyces* medium for 20 hr were washed with water and treated with glutaraldehyde at $0^{o}C$ for 30 min as described in Section 5-2-1. After prefixation, cells were extensively washed with water, sucrose-AMT buffer (sucrose 0.5M, NH_4Cl 0.1M, $MgCl_2$ 0.01M, Tris HCl 0.01M pH 7.6), disrupted by homogenization with glass beads and fractionated as described in Section 4-2-2. The mitochondrial fraction and cytoplasmic ribosomes were prepared according to method C of Section 4-2-3 and Section 4-2-4. Nucleic acids were extracted from these subcellular fractions by phenol-SDS, and analyzed by electrophoresis in 2.4% polyacrylamide gels for 4 hr at room temperature. (A) and (B) are electrophoretic patterns of nucleic acids of mitochondria and cytoplasmic rRNA respectively. (C) is the pattern of a mixture of mitochondrial nucleic acids and cytoplasmic rRNA.

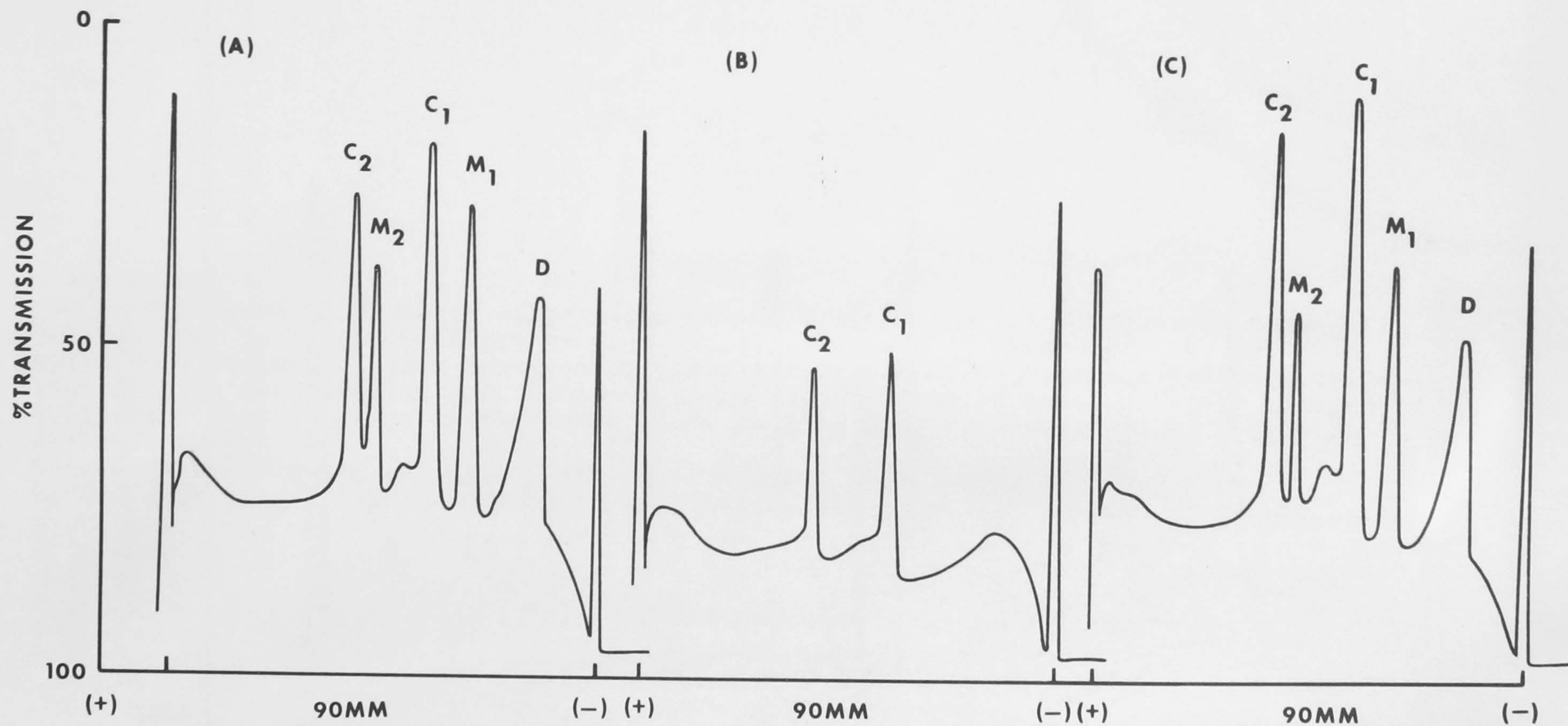
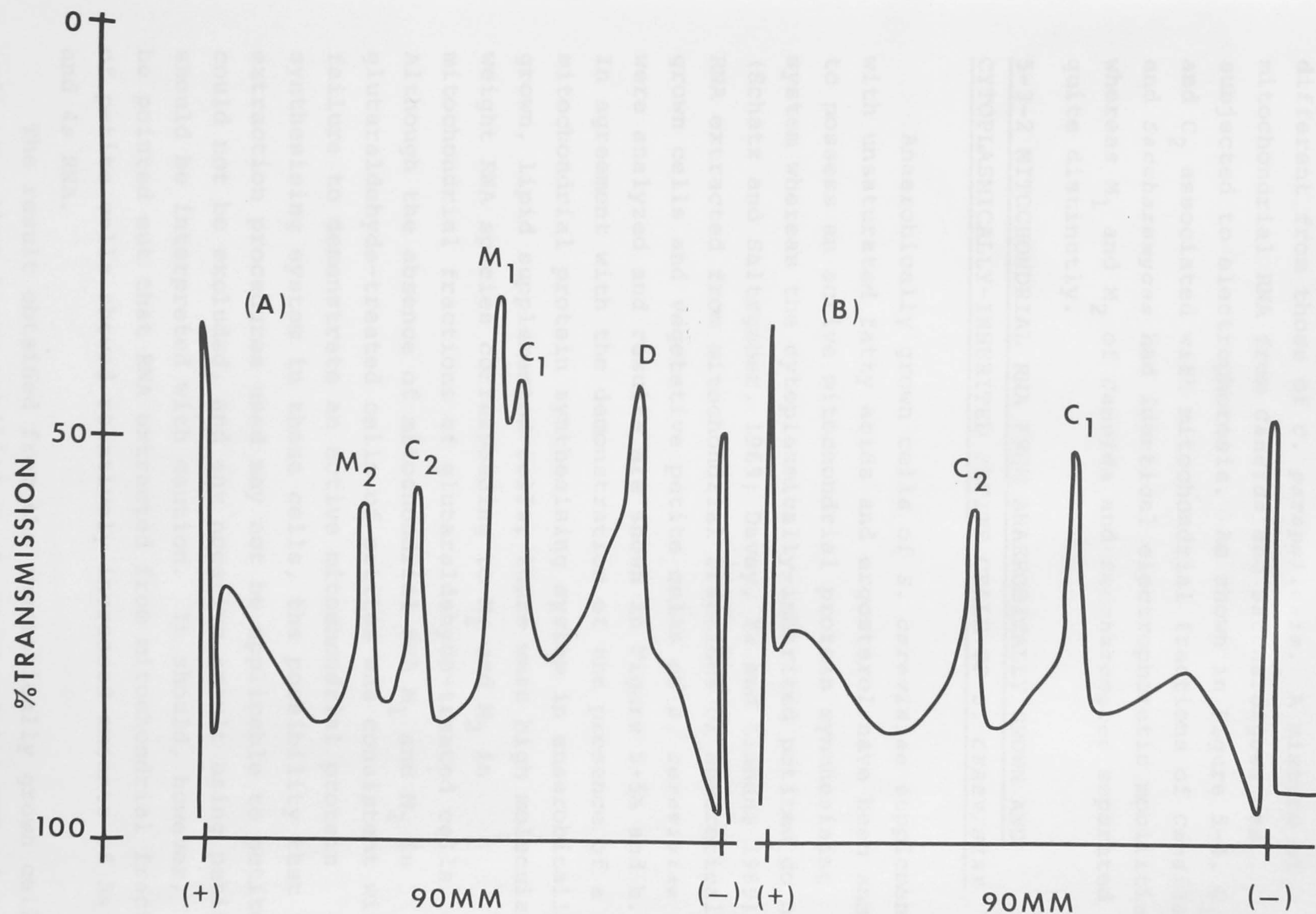

0
50
100
%TRANSMISSION
(A)
(B)
(C)
C2
M2
C1
M1
D
(+)
90MM
(–)

Figure 5-3. Electrophoretic patterns of mitochondrial nucleic acids and cytoplasmic ribosomal RNA of *C. parapsilosis* after prefixation of cells with glutaraldehyde.
Cells of a culture of *C. parapsilosis* grown in 2.5% glucose *Saccharomyces* medium to time T=16 hr were treated with glutaraldehyde as described in Section 5-2-1. Nucleic acids were extracted from subcellular fractions of the glutaraldehyde-prefixed cells and analyzed by electrophoresis in gels for 4 hr at room temperature. (A) and (B) are electrophoretic patterns of mitochondrial nucleic acids and cytoplasmic rRNA respectively.

0+



0
50
100
%TRANSMISSION
(A)
M2
C2
M1
C1
D
(B)
C2
C1
(+)
90MM
(−)
(+)
90MM
(−)

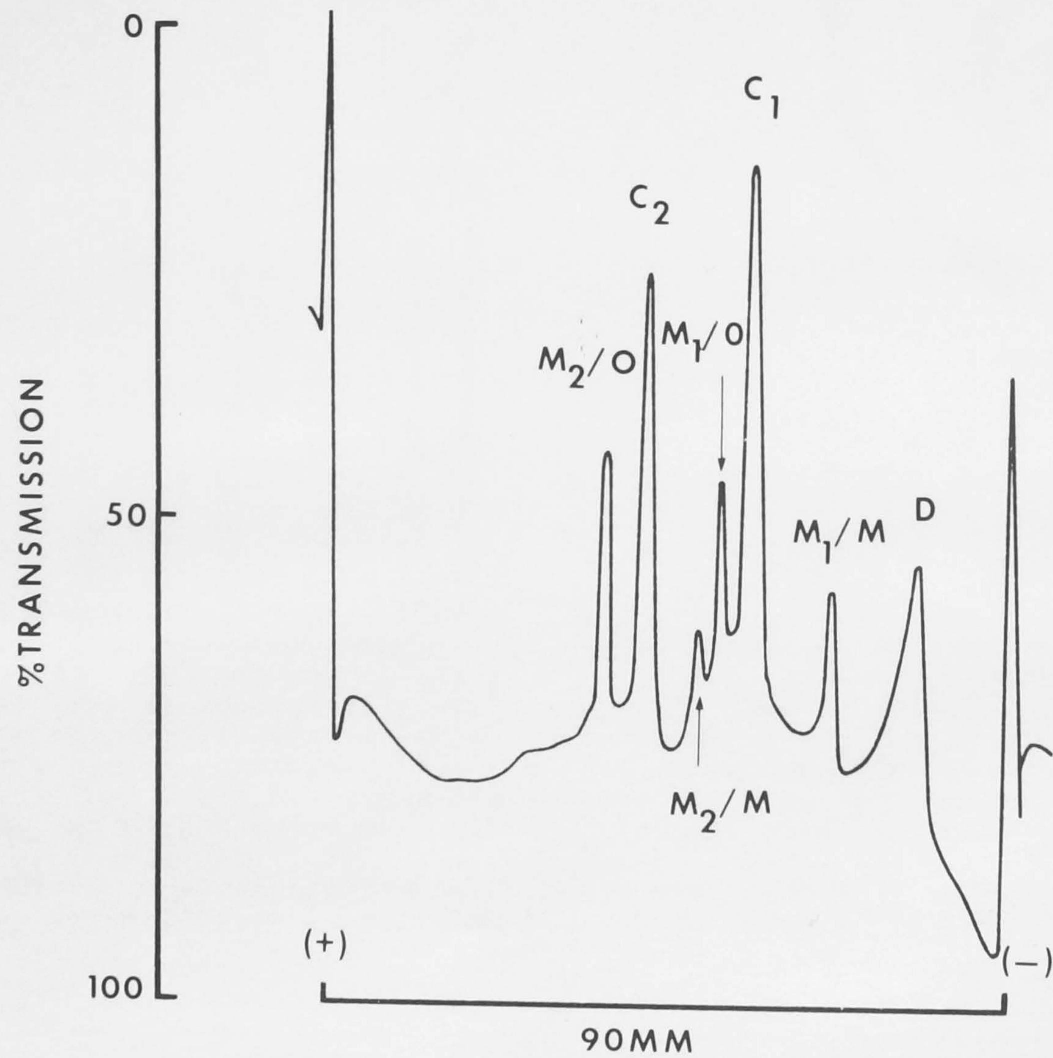
the lower mobilities of mitochondrial RNA of *S. cerevisiae* are intrinsic properties of these molecules and thus different from those of *C. parapsilosis*. A mixture of mitochondrial RNA from *Candida* and *Saccharomyces* was subjected to electrophoresis. As shown in Figure 5-4, C_1 and C_2 associated with mitochondrial fractions of *Candida* and *Saccharomyces* had identical electrophoretic mobilities whereas M_1 and M_2 of *Candida* and *Saccharomyces* separated quite distinctly.

5-3-2 MITOCHONDRIAL RNA FROM ANAEROBICALLY GROWN AND CYTOPLASMICALLY-INHERITED PETITE CELLS OF *S. CEREVISIAE*

Anaerobically grown cells of *S. cerevisiae* supplemented with unsaturated fatty acids and ergosterol have been shown to possess an active mitochondrial protein synthesising system whereas the cytoplasmically-inherited petites do not (Schatz and Saltzgaber, 1969; Davey, Yu and Linnane 1969). RNA extracted from mitochondrial fractions of anaerobically grown cells and vegetative petite cells of *S. cerevisiae* were analyzed and results are shown in Figure 5-5a and b. In agreement with the demonstration of the presence of a mitochondrial protein synthesising system in anaerobically grown, lipid supplemented cells, there were high molecular weight RNA species corresponding to M_1 and M_2 in mitochondrial fractions of glutaraldehyde-treated cells. Although the absence of mitochondrial RNA M_1 and M_2 in glutaraldehyde-treated cells of petites was consistent with failure to demonstrate an active mitochondrial protein synthesising system in these cells, the possibility that the extraction procedures used may not be applicable to petites could not be excluded, and any negative result using petites should be interpreted with caution. It should, however, be pointed out that RNA extracted from mitochondrial fractions of petite cells showed relatively increased amounts of 5*s* and 4*s* RNA.

The result obtained from the anaerobically grown cells indicates that lower mobilities of mitochondrial RNA of *S. cerevisiae* is an intrinsic characteristic of the species and is unaffected by variation of physiological conditions such as anaerobiosis.

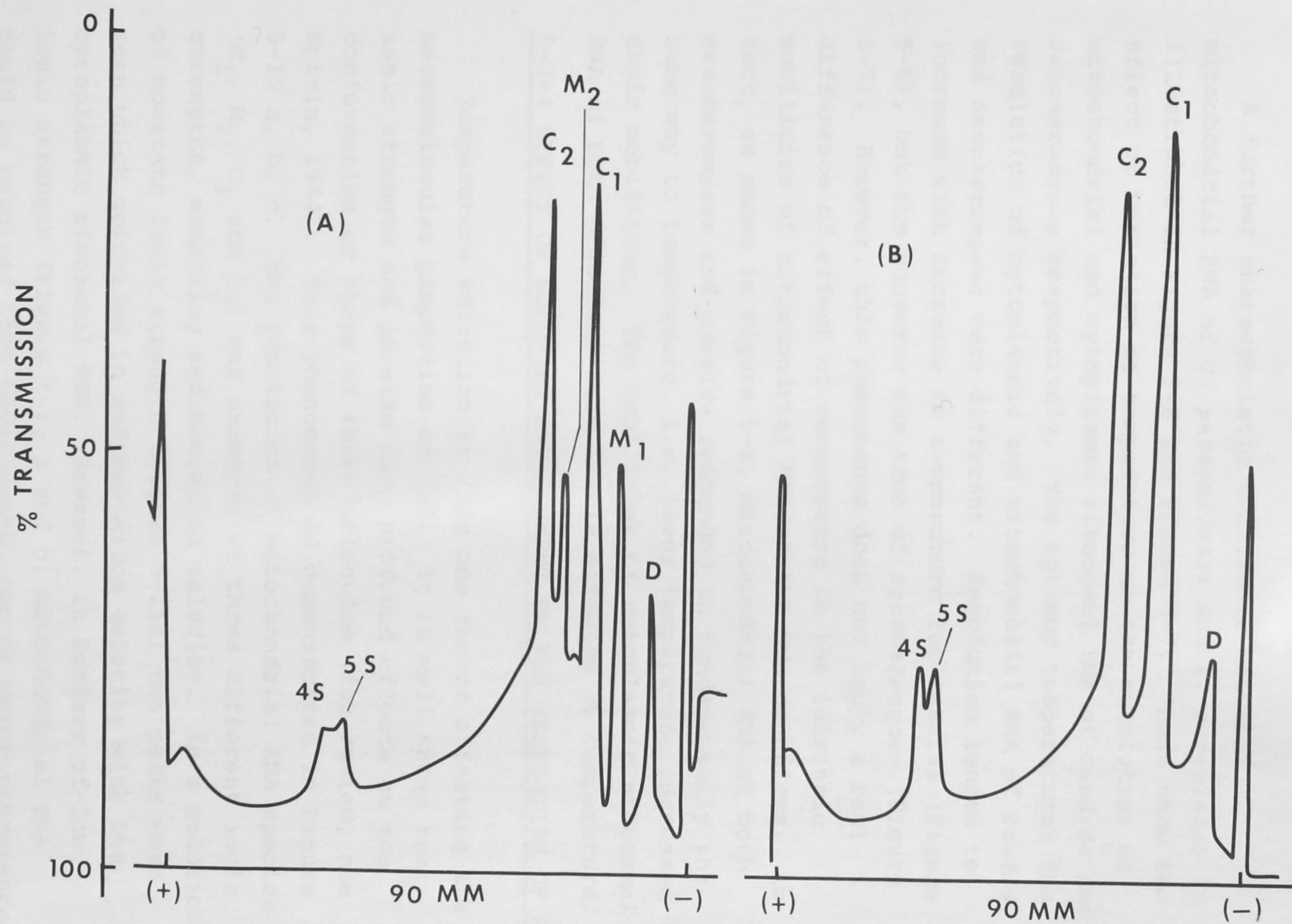
Figure 5-4. The electrophoretic pattern of a mixture of mitochondrial nucleic acids of *C. parapsilosis* and *S. cerevisiae*.
Mitochondrial nucleic acids of *C. parapsilosis* and *S. cerevisiae* were mixed and applied to a polyacrylamide gel for analysis by electrophoresis at room temperature for 4 hr. C_1 and C_2 are cytoplasmic rRNA; M_1/M and M_2/M are mitochondrial RNA of *S. cerevisiae*; and M_1/0 and M_2/0 are mitochondrial RNA of *C. parapsilosis*. Cells of *C. parapsilosis* and *S. cerevisiae* were similarly treated with glutaraldehyde before fractionation.



0
50
100
% TRANSMISSION
C_1
C_2
M_2/O
M_1/O
M_1/M
D
M_2/M
(+)
(−)
90MM

Figure 5-5. Electrophoretic patterns of nucleic acids extracted from mitochondrial fractions of anaerobically grown cells and cells of a vegetative petite of *S. cerevisiae*.

Anaerobically grown and petite cells were removed from cultures (for details of cultivation, see Sections 5-2-3 and 5-2-4), washed and treated with glutaraldehyde as described in Section 5-2-1. Glutaraldehyde-prefixed cells were then disrupted by glass bead homogenization and fractionated to obtain mitochondrial fractions. Nucleic acids were then extracted from mitochondrial fractions and analyzed by electrophoresis in 2.4% polyacrylamide gels at room temperature for 2 hr. (A) and (B) are electrophoretic patterns of nucleic acids extracted from mitochondrial fractions of anaerobically grown cells and cells of a vegetative petite respectively.



% TRANSMISSION
0
50
100
(A)
(B)
C_2
M_2
C_1
M_1
D
4S
5S
(+)
90 MM
(−)

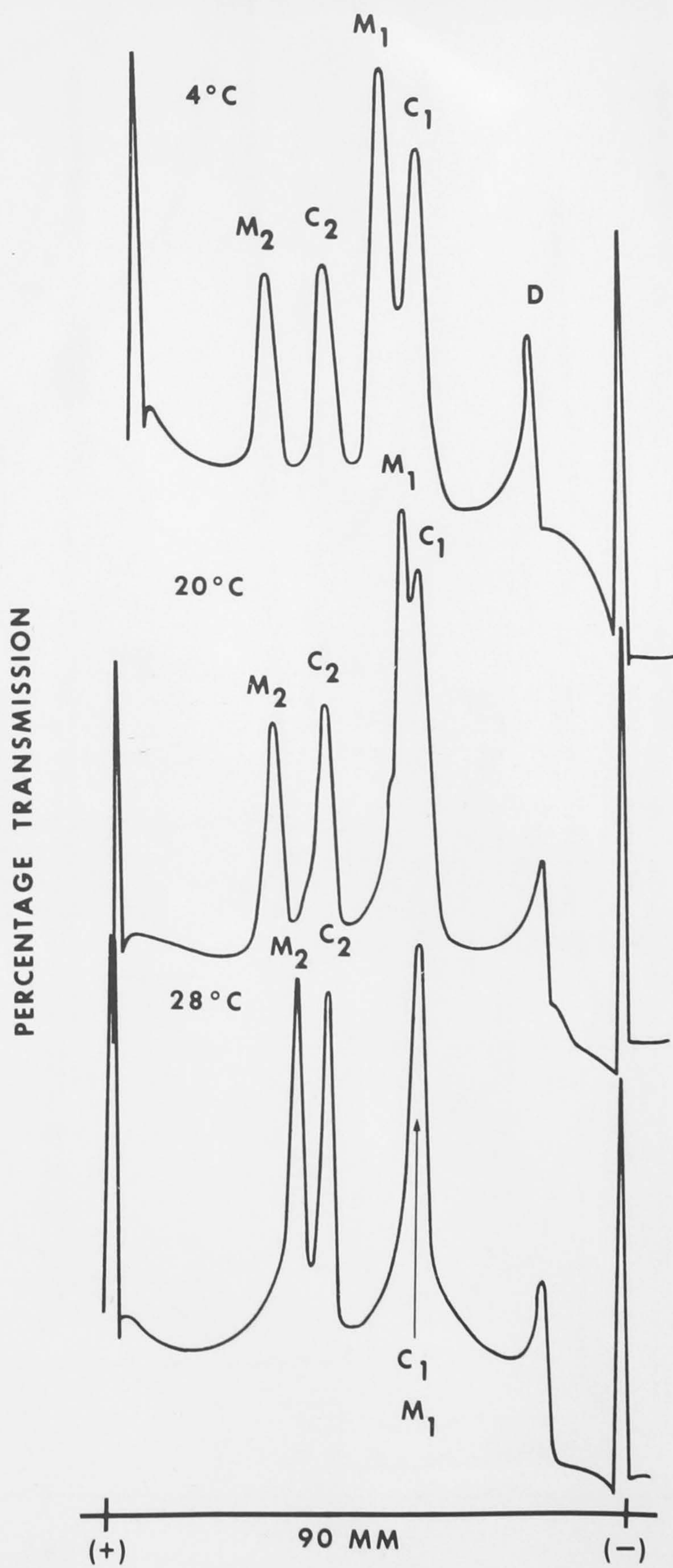
5-3-3 EFFECT OF TEMPERATURE ON ELECTROPHORETIC MOBILITIES OF MITOCHONDRIAL RNA

A further characteristic difference between mitochondrial RNA of *C. parapsilosis* and *S. cerevisiae* is illustrated in Figure 5-6 and Figure 5-7. These show the effect of variation of temperature on the resolution of mitochondrial and cytoplasmic ribosomal RNA of *Candida* and *Saccharomyces* respectively. The optimum temperatures for resolution of cytoplasmic and mitochondrial RNA of *Candida* and *Saccharomyces* were different. Resolution tended to increase with decrease in temperature for *Candida* (Figure 5-6), but the converse was true of *Saccharomyces* (Figure 5-7). However, this phenomenon does not imply a real difference of effect of temperature on the intrinsic mobilities of mitochondrial RNA of the two organisms. In fact, as shown in Figure 5-8, mitochondrial RNA of both *Saccharomyces* and *Candida* responded in fundamentally the same way to temperature, i.e. lower temperature increased their mobilities. The mobilities of cytoplasmic ribosomal RNA of both organisms were little affected by temperature.

5-3-4 EFFECT OF SOLUTION ENVIRONMENT ON THE RESOLUTION OF RNA

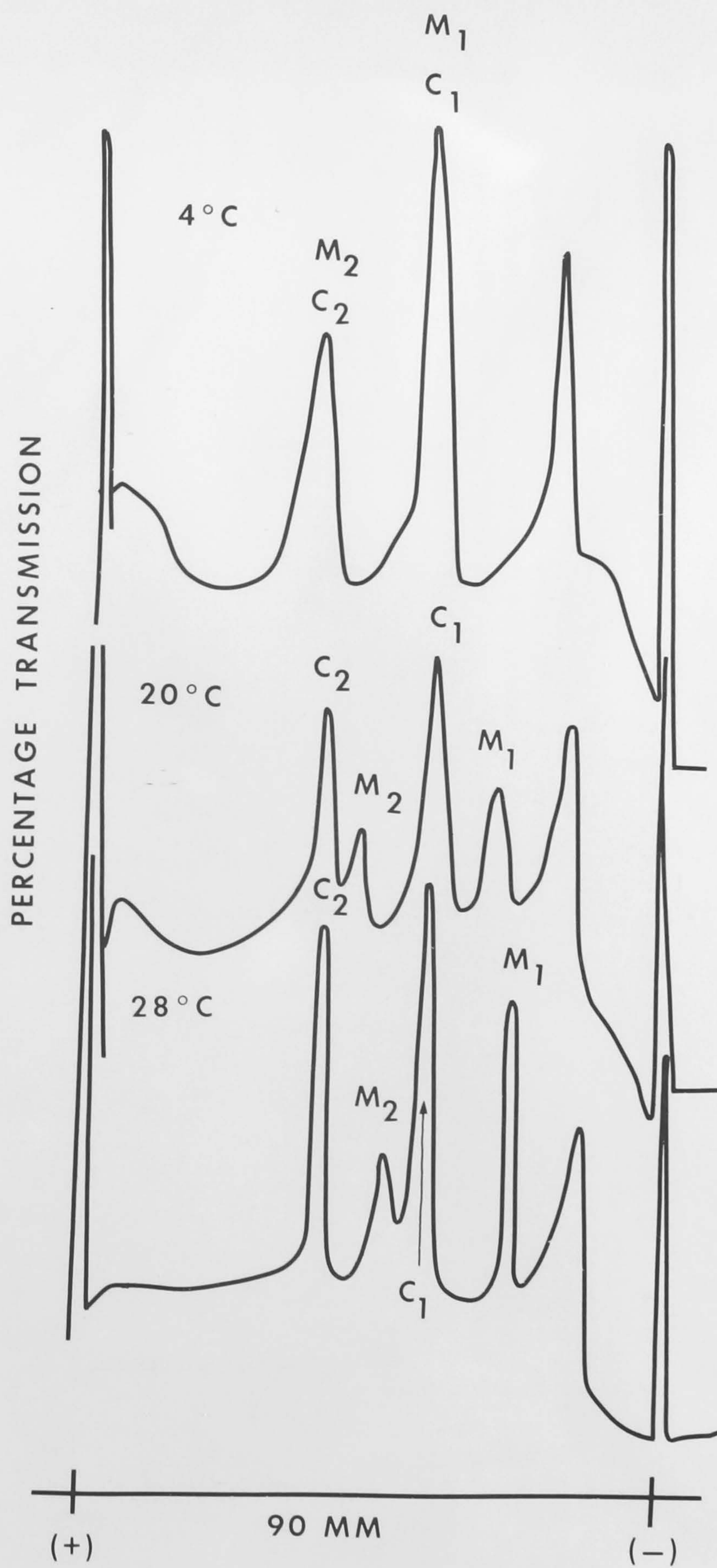
Temperature variation is only one factor affecting the macromolecular properties of RNA. It is well known that ionic strength and pH also have profound effects on the conformation or shape of these molecules (for review, see Spirin, 1964). This phenomenon is demonstrated in Figure 5-15 a, b, c. The resolution of mitochondrial RNA species (M_1, M_2, C_1 and C_2) was examined at three different ionic strengths, measuring sedimentation velocity. In a solution of moderate ionic strength (Figure 5-15a) two peaks were seen which coincided in sedimentation velocity with the cytoplasmic ribosomal RNA. However, in buffers of low ionic strength (Figure 5-15 b and c) mitochondrial RNA could be resolved into three peaks, two of which represented M_2 and C_2 while the third consisted of unresolved M_1 and C_1. The buffer solution of low ionic strength was used by Forrester *et al.* (1971) to separate successfully a mixture

Figure 5-6. Effect of temperature on the resolution of mitochondrial RNA of *C. parapsilosis*. Mitochondrial nucleic acids of *C. parapsilosis* were analyzed by electrophoresis in 2.4% polyacrylamide gels for 4 hr at 4°C, 20°C and 28°C. At 4°C, sodium dodecyl sulphate (SDS) was omitted from the electrophoresis buffer, but control experiments showed that omission of SDS had no effect on the electrophoretic mobilities and resolution of mitochondrial RNA at 20°C and 28°C.



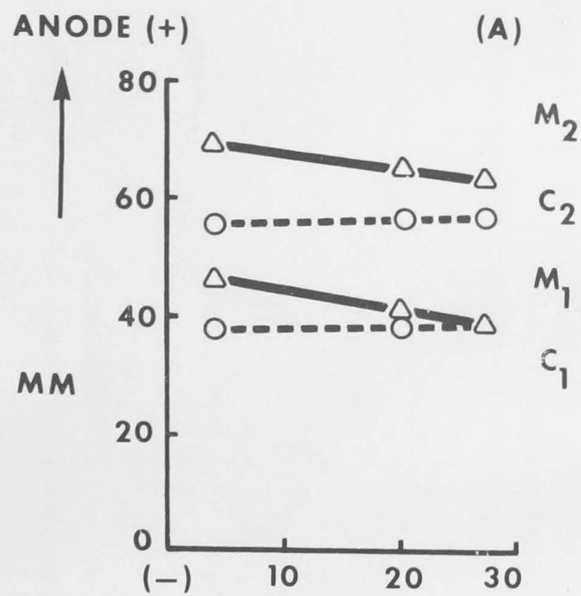
PERCENTAGE TRANSMISSION
4°C
M1
C1
M2
C2
D
20°C
M1
C1
M2
C2
28°C
M2
C2
C1
M1
(+)
90 MM
(−)

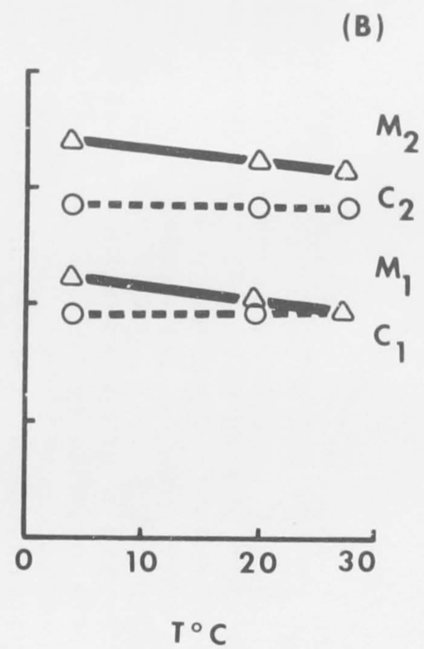
Figure 5-7. Effect of temperature on the resolution of mitochondrial RNA of *S. cerevisiae*. Mitochondrial nucleic acid extracts of *S. cerevisiae* were analyzed by electrophoresis at 4°C, 20°C and 28°C for 4 hr in 2.4% polyacrylamide gels.

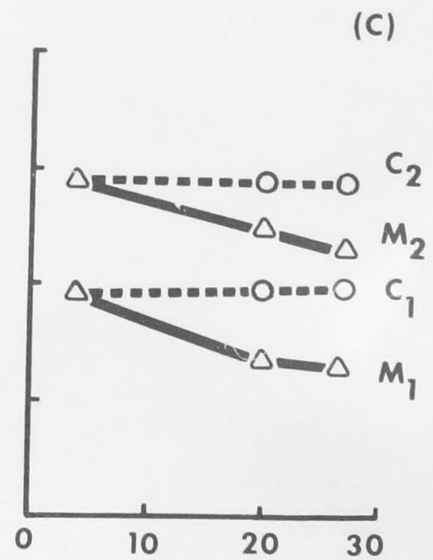


PERCENTAGE TRANSMISSION
4°C
M_1
C_1
M_2
C_2
20°C
C_1
C_2
M_2
M_1
28°C
C_2
M_1
M_2
C_1
90 MM
(+)
(−)

Figure 5-8. Effect of temperature on electrophoretic mobilities of mitochondrial RNA of *C. parapsilosis* and *S. cerevisiae*.
The ordinate represents electrophoretic mobility in mm from the cathode end of the gel (origin). The abscissa represents temperature of the electrophoresis buffer in ^{o}C. Mobilities of individual RNA species were measured at $4^{o}C$, $20^{o}C$ and $28^{o}C$. Graphs of C_1 and C_2 are denoted by O----O and M_1 and M_2 by △——△ respectively. (A), (B) and (C) are graphs of variations of mobilities with temperature of mitochondrial RNA from *C. parapsilosis*, glutaraldehyde-prefixed *C. parapsilosis*, and glutaraldehyde-prefixed *S. cerevisiae* respectively.

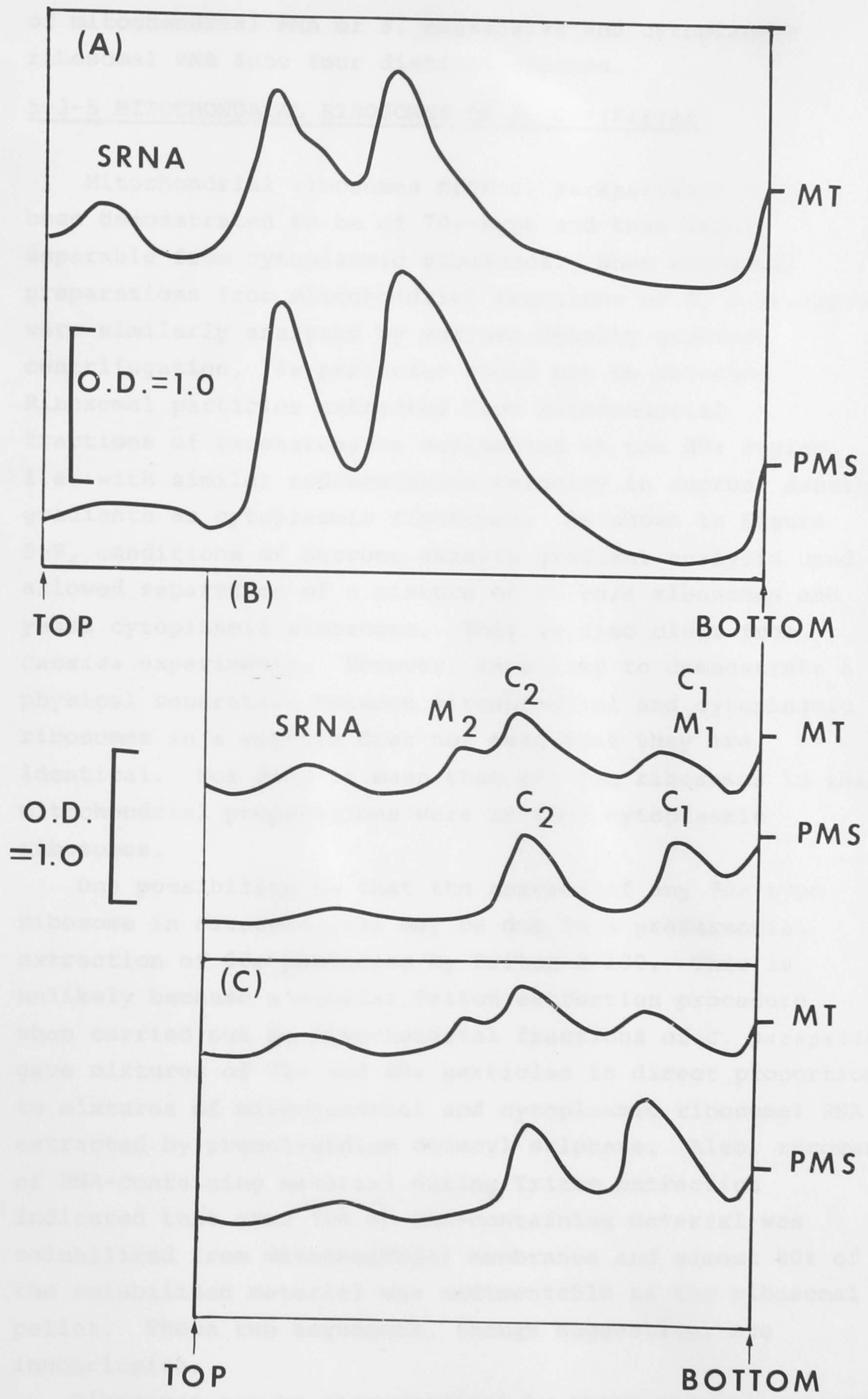






T°C

Figure 5-15. Sedimentation patterns of mitochondrial nucleic acids (MT) and cytoplasmic ribosomal RNA (PMS) of *C. parapsilosis*. Mitochondrial nucleic acids (MT) and cytoplasmic rRNA (PMS) were analyzed in sucrose density gradients, 5-20% w/v in 3 different ionic environments, but under similar conditions of centrifugation, i.e. 27000 rpm for 16 hr at $4^{o}C$ (Spinco, SW 27) with a gradient volume of 36 ml. Sedimentation patterns of RNA were examined at 254 mμ with an ISCO gradient fractionator. (A), (B) and (C) are sedimentation patterns of mitochondrial and cytoplasmic ribosomal RNA in E buffer (Tris acetate 0.04M, sodium acetate 0.02M, sodium EDTA 0.001M pH 7.8), TK buffer (KCl 0.005M, Tris HCl 0.015M pH 7.4) and TKE buffer (KCl 0.005M, Tris HCl 0.015M pH 7.4, sodium EDTA 0.001M pH 7.4) respectively.


(A)
SRNA
MT
O.D.=1.0
PMS
TOP
(B)
BOTTOM
SRNA
M2
C2
C1
M1
MT
O.D.
=1.0
C2
C1
PMS
(C)
MT
PMS
TOP
BOTTOM

of mitochondrial RNA of *S. cerevisiae* and cytoplasmic ribosomal RNA into four distinct species.

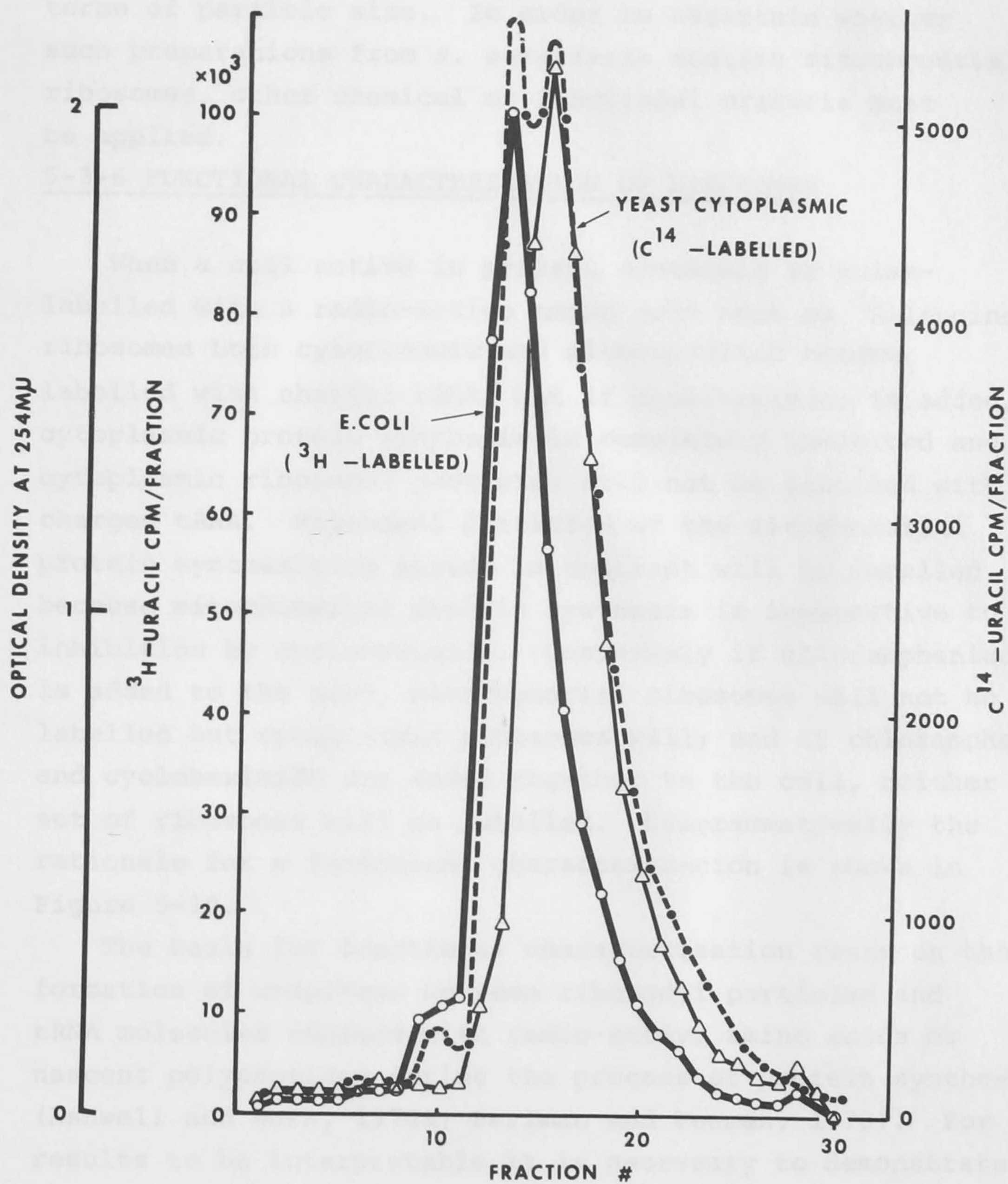
5-3-5 MITOCHONDRIAL RIBOSOMES OF *S. CEREVISIAE*

Mitochondrial ribosomes from *C. parapsilosis* have been demonstrated to be of 70*s*-type and thus easily separable from cytoplasmic ribosomes. When ribosomal preparations from mitochondrial fractions of *S. cerevisiae* were similarly analyzed by sucrose density gradient centrifugation, 70*s* particles could not be detected. Ribosomal particles extracted from mitochondrial fractions of *Saccharomyces* sedimented at the 80*s* region, i.e. with similar sedimentation velocity in sucrose density gradients as cytoplasmic ribosomes. As shown in Figure 5-9, conditions of sucrose density gradient analysis used allowed separation of a mixture of *E. coli* ribosomes and yeast cytoplasmic ribosomes. This is also clear from *Candida* experiments. However, inability to demonstrate a physical separation between mitochondrial and cytoplasmic ribosomes in a mixture does not mean that they are identical. Nor does it mean that all the ribosomes in the mitochondrial preparations were in fact cytoplasmic ribosomes.

One possibility is that the absence of any 70*s* type ribosome in *Saccharomyces* may be due to a preferential extraction of 80*s* particles by Triton X-100. This is unlikely because a similar Triton extraction procedure when carried out on mitochondrial fractions of *C. parapsilosis* gave mixtures of 70*s* and 80*s* particles in direct proportion to mixtures of mitochondrial and cytoplasmic ribosomal RNA extracted by phenol-sodium dodecyl sulphate. Also, recovery of RNA-containing material during Triton extraction indicated that over 70% of RNA-containing material was solubilized from mitochondrial membranes and almost 80% of the solubilized material was sedimentable as the ribosomal pellet. These two arguments, though suggestive, are inconclusive.

Ribosomes can be characterized by three general criteria, viz., physical, chemical and functional. Failure to achieve

Figure 5-9. Resolution of a mixture of *E. coli* and yeast cytoplasmic ribosomes by sedimentation in a sucrose density gradient.
Ribosomes of *E. coli* were labelled by growing cells in uracil-6-^{3}H (36.9 Ci/mmole) at 200 μCi per litre of culture for 16 hr, and ribosomes of *S. cerevisiae* were labelled by growing cells in uracil-2-C^{14} (54.9 mCi/mmole), at 50 μCi per litre of 1% glucose *Saccharomyces* medium for 16 hr. Cells of *E. coli* and *S. cerevisiae* were washed, disrupted and fractionated to obtain ribosomal fractions. The ribosomal fractions of *E. coli* and yeast were mixed (10 O.D. $_{260\ m\mu}$ of *E. coli* ribosomes + 10 O.D. $_{260\ m\mu}$ of yeast ribosomes) and the mixture was analyzed by centrifugation in a sucrose density gradient, 10-20% (w/v) sucrose in AMT at 25000 rpm for 4 hr (Spinco, SW 25.1). The resultant gradient was fractionated and radio-activities of fractions were determined as described in Section 5-2-2. ○——○, ^{3}H radio-activity; △——△, C^{14} radio-activity; ●----●, absorbance as measured by the ISCO gradient fractionator at 254 mμ.


OPTICAL DENSITY AT 254MU
0
1
2
3H-URACIL CPM/FRACTION
×10³
0
10
20
30
40
50
60
70
80
90
100
C14-URACIL CPM/FRACTION
0
1000
2000
3000
4000
5000
YEAST CYTOPLASMIC
(C14 -LABELLED)
E.COLI
(3H -LABELLED)
10
20
30
FRACTION #

physical separation of mitochondrial and cytoplasmic ribosomes in *S. cerevisiae* (especially in apparently contaminated preparations) is *sensu stricto* a negative result and could not constitute an argument that mitochondrial and cytoplasmic ribosomes are the same in terms of particle size. In order to ascertain whether such preparations from *S. cerevisiae* contain mitochondrial ribosomes, other chemical or functional criteria must be applied.

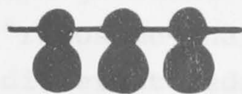
5-3-6 FUNCTIONAL CHARACTERIZATION OF RIBOSOMES

When a cell active in protein synthesis is pulse-labelled with a radio-active amino acid such as ^{3}H-leucine, ribosomes both cytoplasmic and mitochondrial become labelled with charged tRNA; but if cycloheximide is added, cytoplasmic protein synthesis is completely inhibited and cytoplasmic ribosomal particles will not be labelled with charged tRNA. Ribosomal particles of the mitochondrial protein synthesising system in contrast will be labelled, because mitochondrial protein synthesis is insensitive to inhibition by cycloheximide. Conversely if chloramphenicol is added to the cell, mitochondrial ribosomes will not be labelled but cytoplasmic ribosomes will; and if chloramphenicol and cycloheximide are added together to the cell, neither set of ribosomes will be labelled. Diagrammatically the rationale for a functional characterization is shown in Figure 5-10.

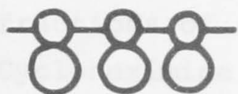
The basis for functional characterization rests on the formation of complexes between ribosomal particles and tRNA molecules charged with radio-active amino acids or nascent polypeptides during the process of protein synthesis (Ashwell and Work, 1970a; Perlman and Penman, 1970). For results to be interpretable it is necessary to demonstrate that complexes are (1) not random adsorption of charged tRNA to ribosomes, and (2) once formed, stable to experimental processing. Test by functional characterization is a criterion *per se,* independent of any physical or chemical prerequisites. The test by itself can only indicate if a ribosomal species of interest is present. However, in

Figure 5-10. Rationale for the functional characterization of ribosomes.
Ribosomes inside the outer perimeter but outside the inner perimeter represent cytoplasmic ribosomes. Ribosomes inside the inner perimeter represent mitochondrial ribosomes. The outer and inner perimeters represent the boundary of a yeast cell and the membrane boundary of a mitochondrion.

DIAGRAMATIC RATIONALIZATION OF FUNCTIONAL CHARACTERIZATION OF RIBOSOMES

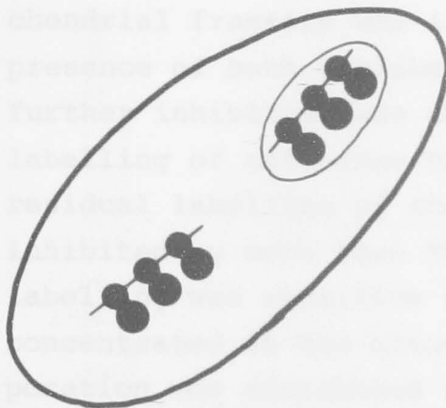


RIBOSOMAL PARTICLES LABELLED WITH RADIOACTIVE AMINO ACID AND/OR NASCENT POLYPEPTIDES VIA t-RNA

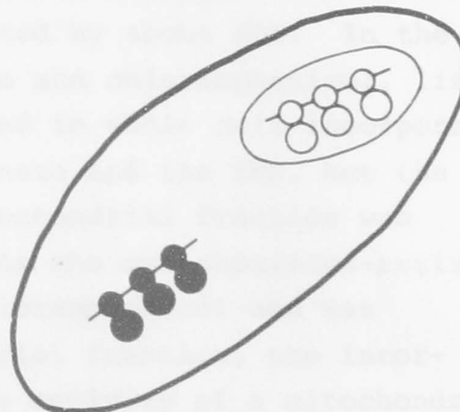


RIBOSOMAL PARTICLES NOT LABELLED

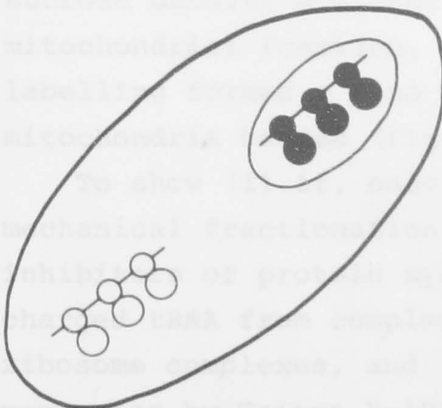
CONTROL+H3-LEUCINE



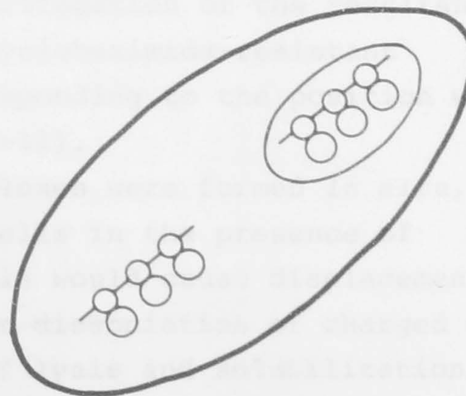
+CAP+H3-LEUCINE



+CHI+H3-LEUCINE



+CHI+CAP+H3-LEUCINE



conjunction with physical characterization such as sucrose density gradient analysis, functional characterization may be used to obtain an estimate of size of the ribosome. It should be emphasized that when functional characterization is used in conjunction with analysis by sucrose density gradient centrifugation, the particles being analysed in the gradient are not ribosomal particles *per se* but "ribosome-charged tRNA-mRNA complexes" which may have a different sedimentation velocity compared with monoribosomes of bipartite structure.

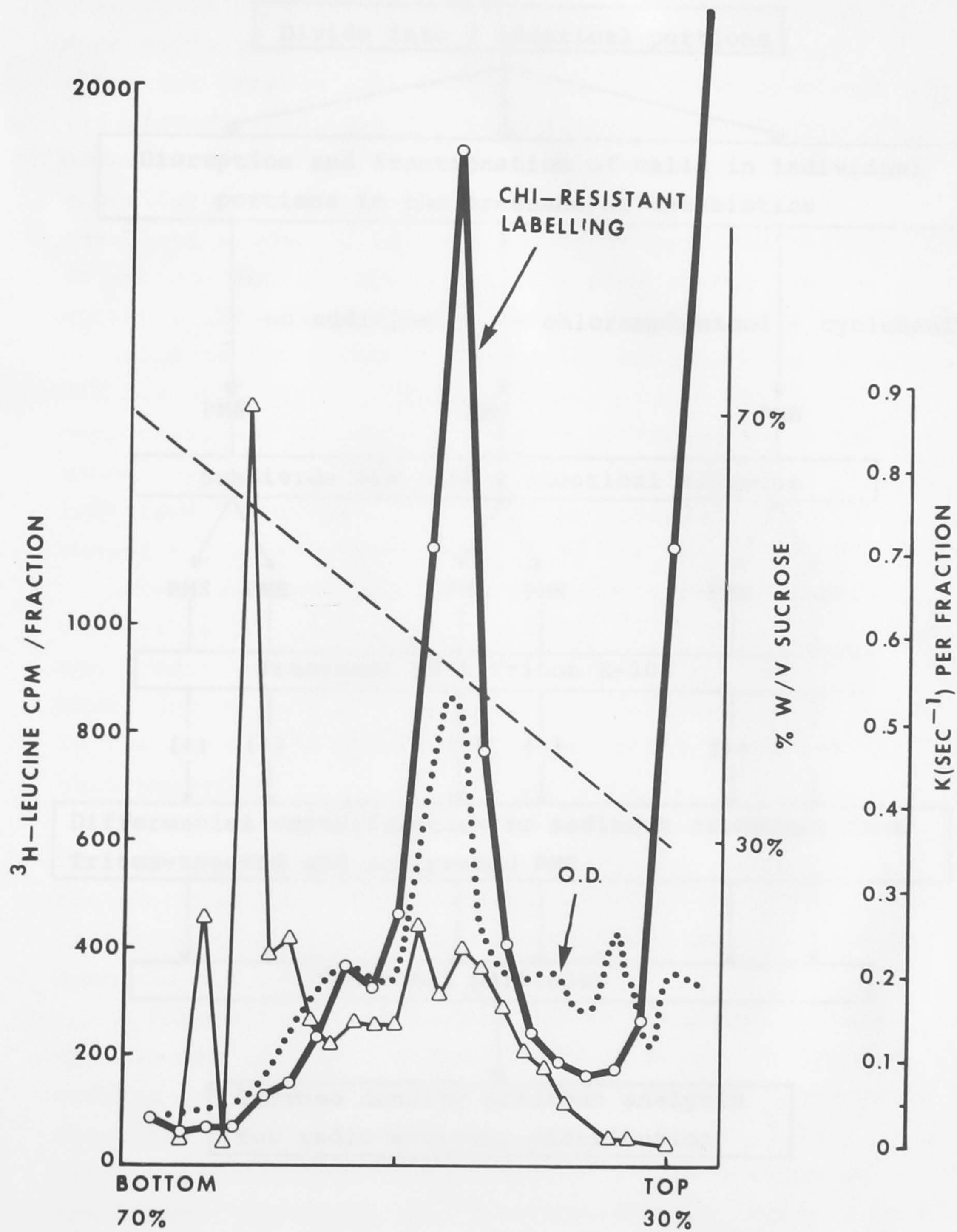
Pulse-labelled with ^{3}H-leucine for 10 min, subcellular fractions of *S. cerevisiae* became labelled (Table 5-1). Cycloheximide severely inhibited labelling of all subcellular fractions. Over 90% inhibition was observed of incorporation into whole cells, and labelling of cell-free homogenate and of the postmitochondrial supernatant (PMS). The mitochondrial fraction was inhibited by about 80%. In the presence of both cycloheximide and chloramphenicol, little further inhibition was observed in whole cell incorporation, labelling of cell-free homogenate and the PMS, but the residual labelling of the mitochondrial fraction was inhibited by more than 80%. As the cycloheximide-resistant labelling was sensitive to chloramphenicol and was concentrated in the mitochondrial fraction, the incorporation was attributed to the activity of a mitochondrial protein synthesising system (see chapter 3 for definition of *in vivo* assessment of mitochondrial protein synthesis). On sucrose density gradient centrifugation of the resultant mitochondrial fraction, the cycloheximide-resistant labelling formed a zone corresponding to the position where mitochondria banded (Figure 5-11).

To show (1) if, once complexes were formed *in situ*, mechanical fractionation of cells in the presence of inhibitors of protein synthesis would cause displacement of charged tRNA from complexes or dissociation of charged tRNA-ribosome complexes, and (2) if lysis and solubilization of membranes by Triton X-100 would cause randomization and dissociation of charged tRNA-ribosome complexes, the following experiments were carried out. Cells were pulse-

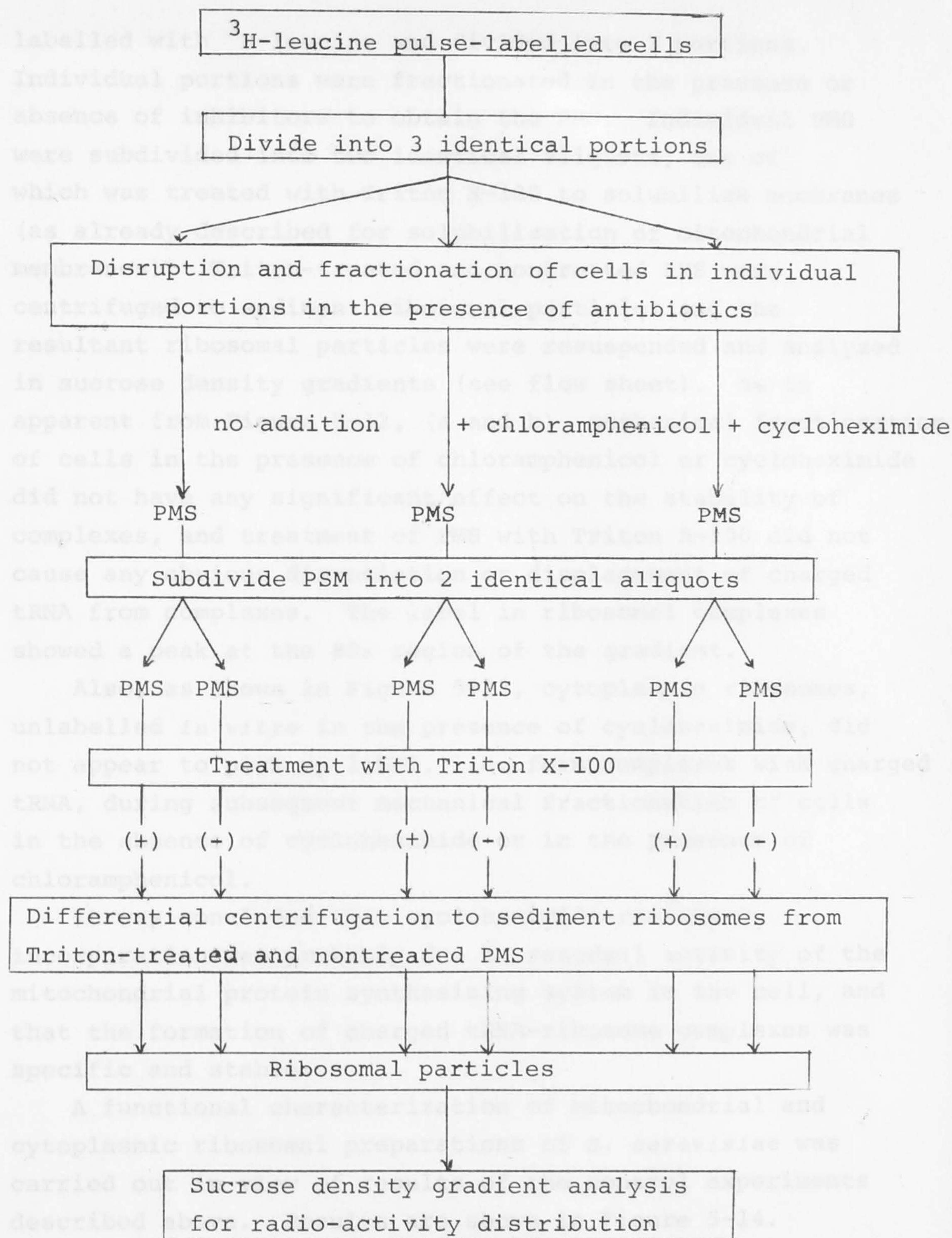
INHIBITOR ADDED	WHOLE CELL INCORPORATION (cpm/unit 640 mμ)	INCORPORATION INTO SUBCELLULAR FRACTION (cpm/mg protein)		
		WH	MT	PMS
NIL	860(O)	2690(O)	3820(O)	2720(O)
+CHI	59(93)	156(94)	668(83)	75(97)
+CHI+CAP	25(97)	46(98)	84(98)	59(98)

Table 5-1. Incorporation into subcellular fractions of *S. cerevisiae* after pulse-labelling with leucine-4-5-^{3}H (34.1 mCi/μmole).
Cells of *S. cerevisiae* cultivated in 1.0% glucose *Saccharomyces* medium for 20 hr were washed and equilibrated with appropriate antibiotics in glucose-phosphate buffer for 15 min. They were then pulse-labelled with leucine-4-5-^{3}H at 50 μCi per 200 ml of cell suspension (containing 2 gm dry wt of cells) for 10 min. Antibiotics were used at concentrations of 100 μg/ml and 3 mg/ml for CHI and CAP respectively. After pulse-labelling, cells were immediately chilled, centrifuged and washed. Samples of these cells were processed for determination of incorporation. Washed cells were also fractionated to obtain subcellular fractions, mitochondria (MT) and postmitochondrial supernatant (PMS), from the cell-free homogenate (WH). These subcellular fractions were then processed for incorporation of radio-active leucine. Details of experimental procedures are described in Section 5-2-2. Figures in brackets are percentage inhibition by antibiotics of incorporation.

Figure 5-11. Isokinetic banding of ^{3}H-leucine labelled mitochondria in a sucrose density gradient. Mitochondria labelled *in vivo* with leucine-4-5-^{3}H in the presence of CHI (100 μg/ml) as described in Section 5-2-2 and in Table 5-1 were sedimented by centrifugation at 24000 rpm for 1 hr at 4°C (Spinco, SW 25.1) through a sucrose density gradient of 30-70% (w/v) sucrose in AMT (NH_4Cl 0.1M, $MgCl_2$ 0.01M, Tris HCl 0.01M pH7.6). Mitochondria under these conditions formed a band which was identified by the peak of cytochrome *c* oxidase activity and succinate-cytochrome *c* reductase activity at about 45% sucrose. △——△, distribution of activity of cytochrome *c* oxidase (expressed as k/fraction); ...., distribution of light absorbing material at 254 mμ; ○——○ , distribution of CHI-resistant labelling; -----, sucrose concentration (theoretical).



2000
1000
800
600
400
200
0
3H-LEUCINE CPM /FRACTION
CHI-RESISTANT LABELLING
O.D.
70%
30%
% W/V SUCROSE
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
K(SEC^{-1}) PER FRACTION
BOTTOM
70%
TOP
30%



^{3}H-leucine pulse-labelled cells
Divide into 3 identical portions
Disruption and fractionation of cells in individual portions in the presence of antibiotics
no addition
+ chloramphenicol
+ cycloheximide
PMS
PMS
PMS
Subdivide PSM into 2 identical aliquots
PMS
PMS
PMS
PMS
PMS
PMS
Treatment with Triton X-100
(+)
(-)
(+)
(-)
(+)
(-)
Differential centrifugation to sediment ribosomes from Triton-treated and nontreated PMS
Ribosomal particles
Sucrose density gradient analysis for radio-activity distribution

labelled with ^{3}H-leucine and divided into 3 portions. Individual portions were fractionated in the presence or absence of inhibitors to obtain the PMS. Individual PMS were subdivided into two identical aliquots, one of which was treated with Triton X-100 to solubilize membranes (as already described for solubilization of mitochondrial membranes). Triton-treated and nontreated PMS were centrifuged to sediment ribosomal particles and the resultant ribosomal particles were resuspended and analyzed in sucrose density gradients (see flow sheet). As is apparent from Figure 5-12, (a and b), mechanical fractionation of cells in the presence of chloramphenicol or cycloheximide did not have any significant effect on the stability of complexes, and treatment of PMS with Triton X-100 did not cause any obvious dissociation or displacement of charged tRNA from complexes. The label in ribosomal complexes showed a peak at the 80*s* region of the gradient.

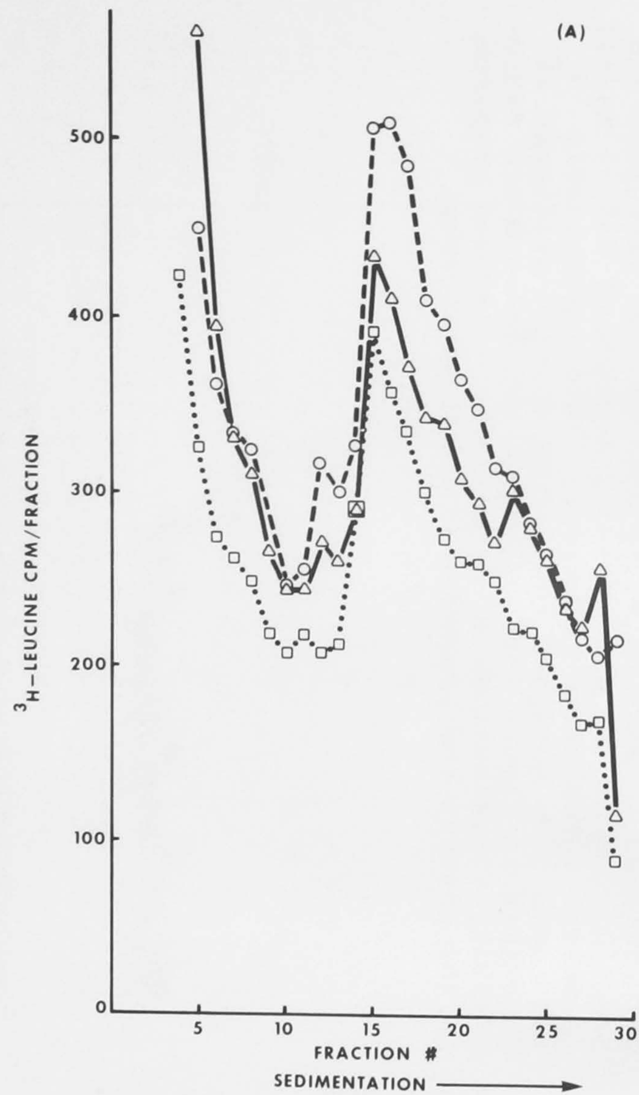
Also, as shown in Figure 5-13, cytoplasmic ribosomes, unlabelled *in vitro* in the presence of cycloheximide, did not appear to pick up label, i.e. form complexes with charged tRNA, during subsequent mechanical fractionation of cells in the absence of cycloheximide or in the presence of chloramphenicol.

It was concluded that cycloheximide-resistant incorporation was probably due to residual activity of the mitochondrial protein synthesising system in the cell, and that the formation of charged tRNA-ribosome complexes was specific and stable.

A functional characterization of mitochondrial and cytoplasmic ribosomal preparations of *S. cerevisiae* was carried out in view of results of the control experiments described above. Results are shown in Figure 5-14. Cytoplasmic ribosomes were labelled in the absence of inhibitor. The radio-activity distribution showed a peak in the 80*s* region of the gradient. This labelling was completely prevented by cycloheximide (Figure 5-14a). Ribosomal particles extracted from mitochondrial fractions were labelled in the presence of cycloheximide, showing a peak of radio-activity in the 80*s* region of the gradient

Figure 5-12. Sedimentation patterns of ^{3}H-leucine labelled cytoplasmic ribosomes.

Cells which were pulse-labelled with leucine-4-5-^{3}H were fractionated in the presence or absence of antibiotics (CAP and CHI) and Triton X-100 to obtain cytoplasmic ribosomes. Cytoplasmic ribosomes prepared under different experimental conditions were then analyzed by centrifugation in sucrose density gradients, 10-20% w/v sucrose in AMT at 25000 rpm for 4 hr at 4°C (Spinco, SW 25.1) for distributions of radioactivity. (A) and (B) are sedimentation patterns of Triton-treated and nontreated cytoplasmic ribosomes respectively. ○----○, △——△, and □····□ are distribution profiles of ^{3}H-leucine labelled ribosomes prepared by homogenization of cells in the absence of antibiotics, in the presence of CHI (100 μg/ml), and in the presence of CAP (3 mg/ml) respectively.


(A)
^{3}H-LEUCINE CPM/FRACTION
500
400
300
200
100
0
5
10
15
20
25
30
FRACTION #
SEDIMENTATION

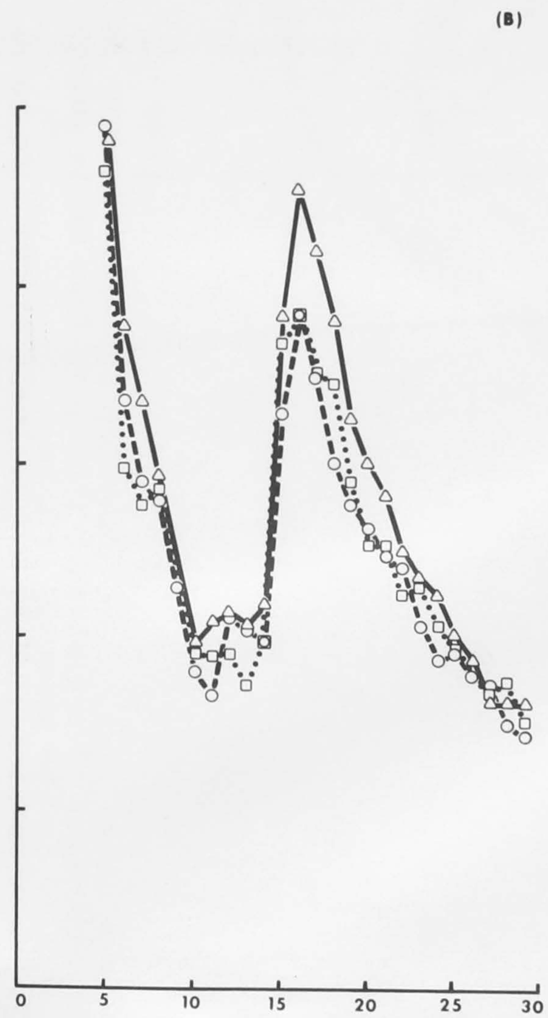

(B)
0
5
10
15
20
25
30

Figure 5-13. Distribution of radio-activity in preparations of cytoplasmic ribosomes from cells pulse-labelled in the presence of CHI.
Cells pulsed labelled in the presence of CHI (100 µg/ml) as described in Section 5-2-2 were washed, disrupted and fractionated in the presence and absence of CAP (3 mg/ml) to obtain cytoplasmic ribosomes. These cytoplasmic ribosomes were analyzed in sucrose density gradients as described in Figure 5-12. ○----○ and ●——● are radio-activity distributions of cytoplasmic ribosomes prepared in the absence and presence of CAP respectively.

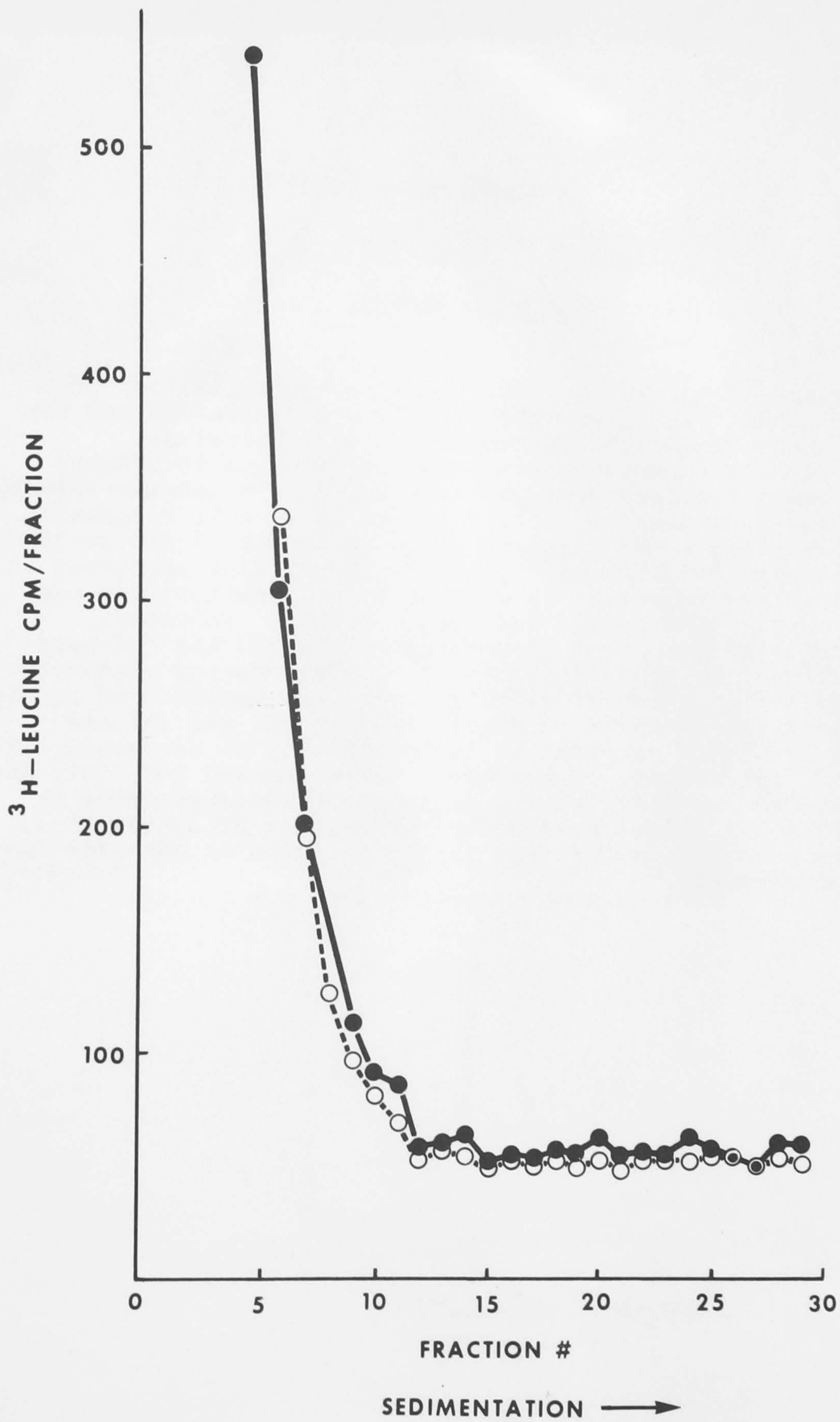
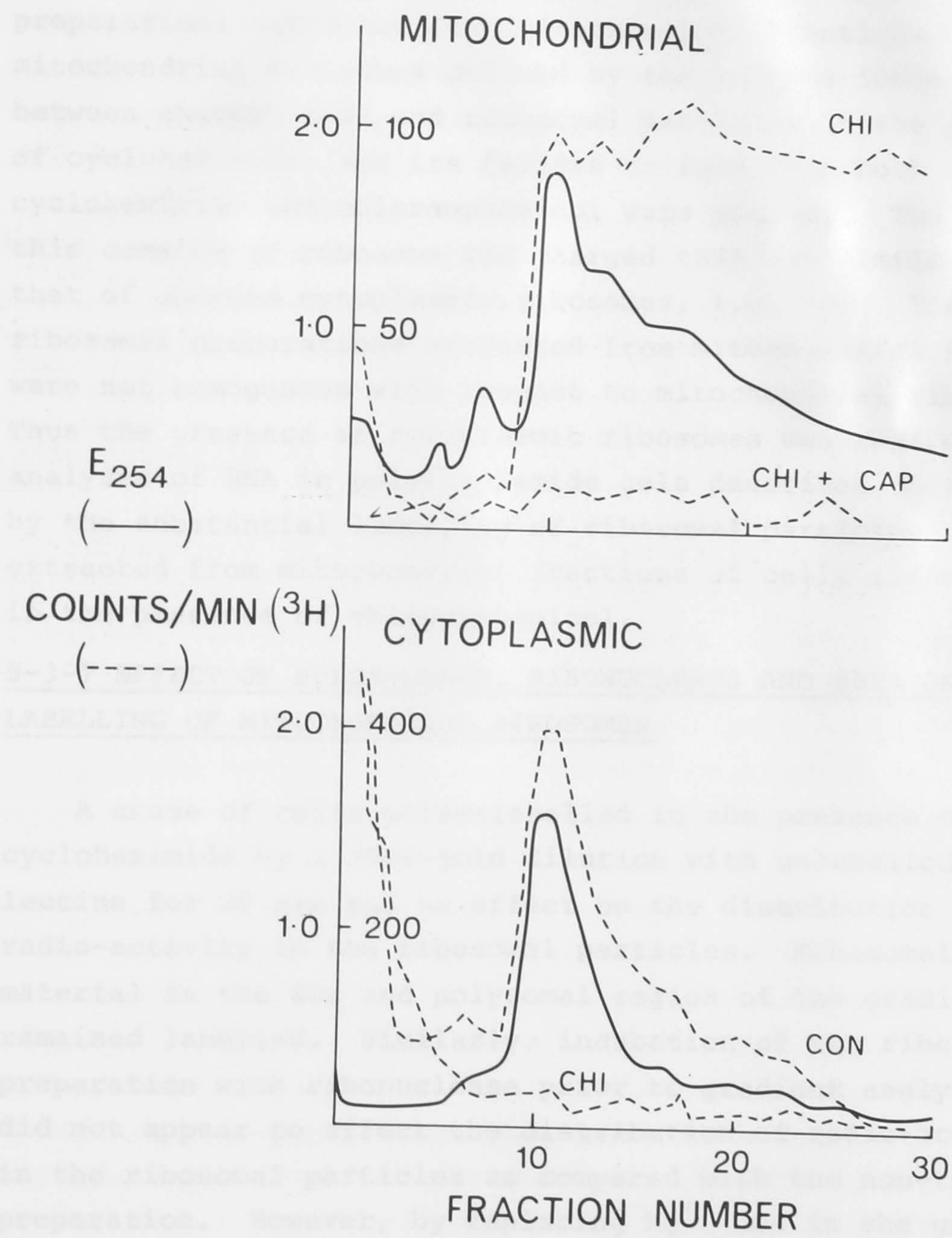

500
400
300
200
100
^{3}H–LEUCINE CPM/FRACTION
0
5
10
15
20
25
30
FRACTION #
SEDIMENTATION

Figure 5-14. Functional characterization of ribosomal particles extracted from mitochondria and the postmitochondrial supernatant of *S. cerevisiae*. Cells after equilibration with antibiotics in glucose phosphate buffer were pulse-labelled with leucine-4-5-^3H (34.1 Ci/mmole) at 50 μCi per 200 ml of cell suspension containing about 2 gm dry wt of cells for 15 min at 29°C and then fractionated to obtain subcellular fractions as described in detail in Section 5-2-2. Antibiotics used were CHI (100 μg/ml) and CAP (3 mg/ml). Ribosomal particles extracted from mitochondria and the PMS were analyzed by centrifugation in sucrose density gradients, and distribution of radio-activity was examined in gradient fractions (details in Figure 5-12). (A) and (B) are distribution patterns of radio-activity in mitochondrial and cytoplasmic ribosomes. Curves marked CON, CHI, and CHI+CAP are distribution patterns of radio-activity in ribosomes pulse-labelled in the absence of antibiotics, in the presence of CHI, and in the presence of CHI plus CAP respectively.



RIBOSOMES FROM
SACCHAROMYCES CEREVISIAE
MITOCHONDRIAL
2·0
100
CHI
1·0
50
E_{254}
(——)
CHI + CAP
COUNTS/MIN (^{3}H)
(-----)
CYTOPLASMIC
2·0
400
1·0
200
CON
CHI
10
20
30
FRACTION NUMBER

and substantial radio-activity was distributed over the polysomal or ribosomal aggregate region of the gradient (Figure 5-14b). This cycloheximide-resistant labelling of ribosomal particles was completely inhibited by simultaneous addition of chloramphenicol and cycloheximide to the cells during pulse-labelling (Figure 5-14b).

It appeared from these results that ribosomal preparations extracted from mitochondrial fractions contained mitochondrial ribosomes defined by the complex formed between charged tRNA and ribosomal particles in the presence of cycloheximide, and its failure to form when both cycloheximide and chloramphenicol were present. The size of this complex of ribosome and charged tRNA was similar to that of charged cytoplasmic ribosomes, i.e. 80*s*. The ribosomal preparations extracted from mitochondrial fractions were not homogenous with respect to mitochondrial ribosomes. Thus the presence of cytoplasmic ribosomes was apparent from analyses of RNA in polyacrylamide gels described above, and by the substantial labelling of ribosomal particles extracted from mitochondrial fractions of cells pulse-labelled in the presence of chloramphenicol.

5-3-7 EFFECT OF PULSE-CHASE, RIBONUCLEASE AND EDTA ON THE LABELLING OF MITOCHONDRIAL RIBOSOMES

A chase of cells pulse-labelled in the presence of cycloheximide by a 2000-fold dilution with unlabelled leucine for 20 min had no effect on the distribution of radio-activity in the ribosomal particles. Ribosomal material in the 80*s* and polysomal region of the gradient remained labelled. Similarly, incubation of the ribosomal preparation with ribonuclease prior to gradient analysis did not appear to affect the distribution of radio-activity in the ribosomal particles as compared with the non-treated preparation. However, by replacing Mg^{++} ion in the gradient buffer with EDTA (1 mM), the distribution of radio-activity in the gradient was changed. The ^{3}H-leucine label dis-appeared in the 80*s* and polysomal region of the gradient, but instead appeared in the 60*s* region of the gradient

(Figure 6-2).

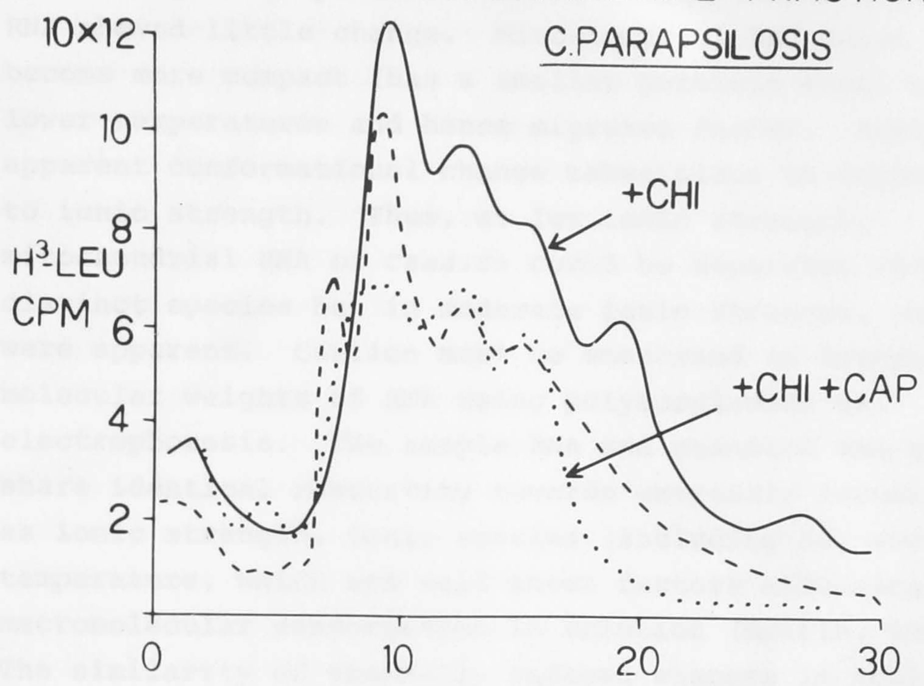
5-3-8 FUNCTIONAL CHARACTERIZATION OF RIBOSOMES OF *CANDIDA PARAPSILOSIS*

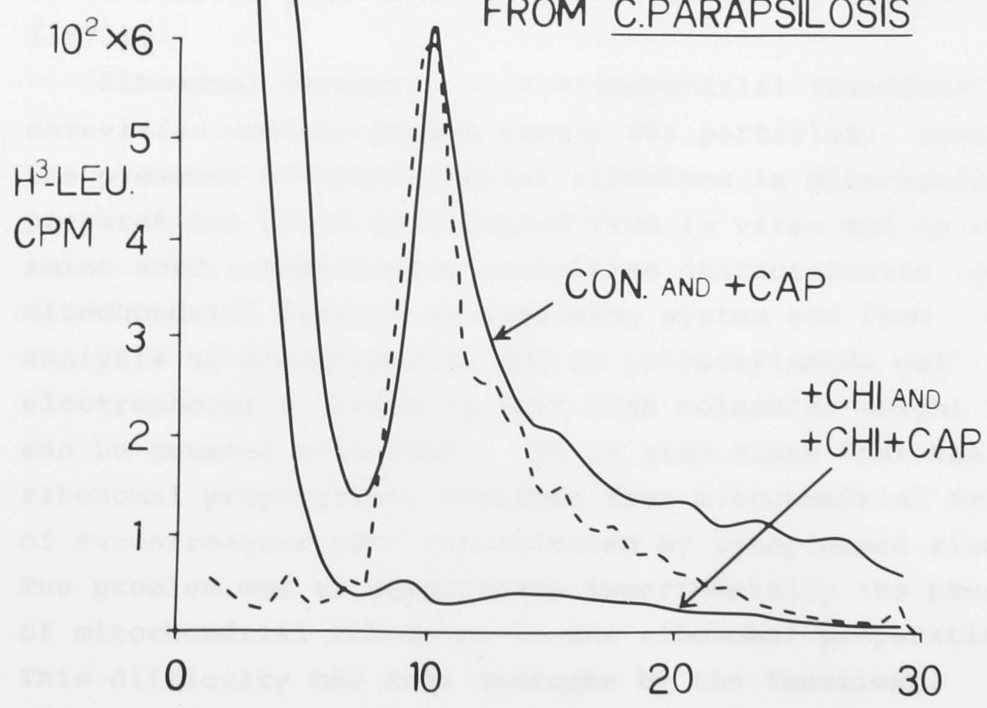
The results of a similar functional characterization of mitochondrial ribosomes from *C. parapsilosis* are shown in Figure 5-16. The optical density trace of the particles in the gradient (Figure 5-16a) shows that there were 70*s* and 80*s* particles as well as some polysomal or ribosomal aggregates. In the presence of cycloheximide, only the 80*s* and polysomal region were labelled; the 70*s* particles were not. The cycloheximide-resistant labelling was inhibited by further addition of chloramphenicol indicating that this labelling was due to activity of the mitochondrial protein synthesising system. Labelling of the cytoplasmic ribosomes was completely inhibited by cycloheximide (Figure 5-16b).

5-4 DISCUSSION

Mitochondrial fractions of *S. cerevisiae* contain species of high molecular weight RNA presumed to be mitochondrial ribosomal RNA on the basis of the number of molecular species present, apparent molecular size, quantity and molar proportion. These preparations also contained cytoplasmic RNA probably derived from contaminating cytoplasmic ribosomes. During electrophoresis on polyacrylamide gel, the two RNA species unique to mitochondria from *S. cerevisiae* migrated more slowly than RNA species from cytoplasmic ribosomes. This was in contrast to mitochondrial RNA species from *C. parapsilosis* which migrated faster than the corresponding cytoplasmic species. Cytoplasmic ribosomal RNA of *Candida* and *Saccharomyces* could not be separated on these gels. The apparent differences between mitochondrial RNA of *Candida* and *Saccharomyces* could reflect conformational differences, rather than differences in molecular weight. However, it is clear that despite this difference in apparent size, mitochondrial RNA of *Candida* and *Saccharomyces* have one property in common that may depend on conformational determinants in these molecules, viz. both showed significant

Figure 5-16. Functional characterization of ribosomal particles extracted from mitochondria and the PMS of *C. parapsilosis*.
Cells of a culture of *C. parapsilosis* grown in 2.5% glucose *Saccharomyces* medium for 20 hr were harvested, washed and pulse-labelled with leucine-4-5-^{3}H in the presence and absence of antibiotics as described in Section 5-2-2. Ribosomal particles were then prepared from pulse-labelled cells and analyzed in sucrose density gradients (see Figure 5-12). (Upper figure) and (Lower figure) are distribution patterns of radio-activity in ribosomes extracted from mitochondria and the PMS respectively. Broken lines (-------) are optical density profiles of ribosomes in gradients. Curves marked CON and + CAP, + CHI and + CHI + CAP, + CHI, and + CHI + CAP are distribution patterns of radio-activity in ribosomal particles labelled in the presence of the appropriate antibiotics.


RIBOSOMAL PREPARATION FROM
MITOCHONDRIAL FRACTION OF
C.PARAPSILOSIS
10×12
10
8
6
4
2
H3-LEU
CPM
+CHI
+CHI +CAP
0
10
20
30


CYTOPLASMIC RIBOSOMES
FROM C.PARAPSILOSIS
10²×6
5
4
3
2
1
H3-LEU.
CPM
CON. AND +CAP
+CHI AND
+CHI+CAP
0
10
20
30

changes of electrophoretic mobilities in response to variations of temperature, whereas cytoplasmic ribosomal RNA showed little change. Mitochondrial RNA seems to become more compact (has a smaller particle size) at lower temperatures and hence migrates faster. Another apparent conformational change takes place in response to ionic strength. Thus, at low ionic strength, mitochondrial RNA of *Candida* could be separated into 3 distinct species but in moderate ionic strength, only two were apparent. Caution must be exercised in determining molecular weights of RNA using polyacrylamide gel electrophoresis. The sample RNA and standard RNA should share identical reactivity towards extrinsic factors such as ionic strength, ionic species (including pH) and temperature, which are well-known factors affecting macromolecular conformation in solution (Spirin, 1964). The similarity of thermally induced changes in mobilities of mitochondrial RNA of *Candida* and *Saccharomyces* might indicate similarities of chemical constitution. Similar results of the effect of temperature and ionic strength on the electrophoretic mobility of mitochondrial RNA of *S. cerevisiae* have recently been reported by Grivell *et al.* (1971).

Ribosomal extracts from mitochondrial fractions of *S. cerevisiae* contain predominantly 80*s* particles. However, the presence of mitochondrial ribosomes in mitochondrial preparations could be inferred from *in vitro* and *in vivo* amino acid incorporating activities characteristic of the mitochondrial protein synthesising system and from analysis of mitochondrial RNA in polyacrylamide gel electrophoresis (assuming that high molecular weight RNA can be equated with rRNA). It is also clear that the ribosomal preparations obtained from mitochondrial fractions of *Saccharomyces* were contaminated by cytoplasmic ribosomes. The problem was to demonstrate experimentally the presence of mitochondrial ribosomes in the ribosomal preparation. This difficulty has been overcome by the functional characterization technique and it could be concluded that there were mitochondrial ribosomal particles in the

preparations. Insofar as cytoplasmic ribosomes charged with labelled tRNA show sedimentation properties similar to those particles isolated without such label, mitochondrial ribosomes are concluded to be 80*s*-type. The cycloheximide-resistant labelling of ribosomal preparations from mitochondria was however unusual in the sense that it was not eliminated by prolonged chase with excess "cold" amino acid and was not digestible by RNase (50 μg/ml) but the labelling peak could be shifted to the 60*s* region of the gradient by EDTA (1mM). The reason for these unusual features of protein synthesis remains to be found.

Functional characterization of mitochondrial ribosomes has been reported by Perlman and Penman (1970). They reported a 95*s* particle for the mitochondrial protein synthesising unit in HeLa cells. The label on the 95*s* particles could however, be chased with unlabelled leucine and could be removed by incubation of pulse-labelled cells with puromycin. They also reported that the label on the 95*s* particles could be shifted to the 40*s* region of the gradient by EDTA treatment.

There have been reports of 80*s* mitochondrial ribosomes in *S. cerevisiae* (Schmitt, 1969; Morimoto and Halvorson, 1970). These reports are in agreement with the results of functional characterization of mitochondrial ribosomes in *Saccharomyces* described above.

A similar experiment was carried out with *Candida*. As shown in Figure 5-16, the cycloheximide-resistant labelling of ribosomal particles extracted from a mitochondrial fraction of *Candida* showed a peak at the 80*s* region but none at the 70*s* region (Figure 5-16a). This cycloheximide-resistant labelling was nevertheless sensitive to inhibition by further addition of chlor-amphenicol. It may therefore be concluded based on the response towards inhibitors that the labelling of ribosomal particles represents mitochondrial protein synthesis *in vivo*. To explain this apparent anomaly, a number of hypotheses can be put forward. For example, the absence of labelling of 70*s* particles of the MPSS may be explained by supposing the mitochondrial protein synthesis of *C. parapsilosis* to be

different from its cytoplasmic protein synthesis as well as mitochondrial and cytoplasmic protein syntheses of *S. cerevisiae*. Mitochondrial ribosomes of *C. parapsilosis* may change their intrinsic particle size when they participate in protein synthesis; they may become labelled with charged tRNA and sediment faster in sucrose density gradients. Direct experimental proof of this hypothesis would require *in vitro* protein synthesis by isolated 70*s* particles in a submitochondrial system.

It is also possible that there is a fundamental difference in the mechanism of cytoplasmic and mitochondrial protein synthesis in *C. parapsilosis* such that mitochondrial protein synthesis is membrane-bound whereas cytoplasmic is not. During protein synthesis, mitochondrial ribosomes may become so firmly attached to membranes such that on solubilization with non-ionic detergents such as Triton X-100, fragments of membranes may still be bound to mitochondrial ribosomes forming quarternary complexes (consisting of ribosome-tRNA-mRNA-membrane fragment). In sucrose density gradient analysis such a complex would sediment faster than a 70*s* particle. The 70*s* particles did not participate in protein synthesis as free ribosomes and hence were not labelled. Experimental proof of this hypothesis would require (1) the demonstration by chemical analysis of an association of marker lipids and/or proteins of membranes with ribosomal particles as well as (2) the visualization by electron microscopy of an association of membrane fragments and ribosomal particles. These remain to be done.

Association of mitochondrial ribosomes and membranes may not be a random physical adsorption but may represent some form of functional interaction such that products of the MPSS may be integrated directly into membranes.

The hypothesis of ribosome attachment to membranes was put forward by Sabatini and Blobel (1970) to explain the observation that in rough microsomes of protein-secretory cells virtually the entire polypeptide is resistant to proteolysis. They envisaged that the ribosomes attach to

the microsomal membrane through the large subunits and the growing polypeptides enter directly into the membrane. The carboxyl-terminal segment is thus protected due to shielding by the large ribosomal subunit, and the amino terminal by the microsomal membrane. Trypsin or chymotrypsin treatment was required to remove ribosomes from rough microsomes indicating a protein linkage between ribosomes and membrane instead of a physical adsorption.

Two conclusions can be drawn from the comparative study of macromolecular components of the mitochondrial protein synthesising system in *Candida* and *Saccharomyces*. Firstly it demonstrated that morphologically and biochemically similar organisms with similar cytoplasmic ribosomes and ribosomal RNA and similar overall mitochondrial protein synthesising activities, may have different mitochondrial ribosomes in terms of their particle size (80*s* in *Saccharomyces* and 70*s* in *Candida*) and RNA constituents; hence as a corollary it demonstrates that similarity of response to inhibitors of mitochondrial protein synthesis is not a consequence of the characteristics of the RNA constituents in the ribosome, nor the size of the ribosome. It will be, therefore, most interesting to compare protein constituents of mitochondrial ribosomes of these two organisms. This may elucidate the problem of the determinant of sensitivity to inhibitors of protein synthesis. Secondly, mechanisms of mitochondrial protein synthesis of *C. parapsilosis* and *S. cerevisiae* may be different although their sensitivities towards antibiotics are similar.

CHAPTER 6

GENERAL DISCUSSION

CHAPTER 6

6-1 GENERAL DISCUSSION

In this chapter, I propose to examine the present state of knowledge concerning two areas of research which are currently receiving much attention. These are the characterization of the mitochondrial protein synthesising system, particularly ribosomes, and the identification of sites of synthesis of mitochondrial components.

6-1-1 SYNTHESIS OF MITOCHONDRIAL COMPONENTS

(i) *In vitro*

Main objectives of studies on mitochondrial differentiation are to identify sites of synthesis of components of mitochondria, how they are brought together and assembled as complex structures, and the regulatory mechanisms controlling these syntheses and assembly. One approach to the site of synthesis of mitochondrial protein and nucleic acid macromolecules, has been to incubate isolated mitochondria in suitable environments in an attempt to achieve an *in vitro* synthesis. The advantage of this approach is that any specific macromolecules synthesized under *in vitro* conditions could be concluded unequivocally to be synthesized by mitochondria *in situ*. This approach, although very simple in concept, has not been successful. Claims (see Ashwell and Work, 1970 for review) have been made that "proteins" synthesized *in vitro* are, or are associated with, structural proteins of the mitochondria. Great interest has been shown in these observations because of the possible directive and organizer role of protein products of the mitochondrial protein synthesising system in the overall development and differentiation of the organelle. More recently, however, it has been suggested that so-called "structural proteins" of mitochondria might be mostly denatured enzymes such as ATPase (see Ashwell and Work, 1970; Schatz, 1970 for review). Careful studies described by Kadenbach (1971) have so far failed to find a

protein product of *in vitro* amino acid incorporation, but instead have revealed a small peptide of lipophilic nature.

Similarly, incubation of isolated mitochondria with labelled nucleotides has been claimed to yield TCA-insoluble material; but the exact nature of these *in vitro* products remains to be defined (see Schatz, 1970 for review).

While there seems no doubt that isolated mitochondria have the capability to incorporate amino acids and nucleotide triphosphates into macromolecules, it seems unlikely on both technical and theoretical grounds that the *in vitro* approach will be very productive. Thus, it will be difficult experimentally to detect and identify the few complete macromolecules synthesized *in vitro*; also it is doubtful whether the mitochondrion excised from the cytoplasm and nucleus could carry enough information to sustain synthesis of sets of macromolecules that may represent structural or functional entities of the organelle.

(ii) *In vivo*

An alternative approach to the dissection of the tangled complexity of interactions that underlies the synthesis of mitochondrial components is the use of specific inhibitors of protein and nucleic acid synthesis in whole cell experiments. This approach has been more successful, but a few criticisms deserve mention. Primary and secondary effects of inhibitors are frequently difficult to separate in this approach, and partial effects are particularly perplexing. Nevertheless this approach has illuminated the field of mitochondrial differentiation substantially.

One of the most notable features of research on mitochondrial differentiation and development over the past few years has been the way in which synthesis of one mitochondrial protein after another has been in turn assigned to cytoplasmic ribosomes (cytochrome *c*, malic dehydrogenase, ATPase, etc) and coded by nuclear genes (see Ashwell and Work, 1970 for review). Considerations of the coding capacity of the mitochondrial genome suggests that there is insufficient information available to encode for many mitochondrial components. Hybridization studies

of mitochondrial RNA (loosely assumed to be mitochondrial ribosomal RNA) with mitochondrial DNA, and inhibitor studies using ethidium bromide indicate that mitochondrial DNA codes for mitochondrial ribosomal RNA. As well as rRNA, mitochondrial DNA may code for up to 20 species of tRNA (for review see Schatz, 1970; Kuntzel, 1971). Thus, it appears that information required to code for these RNA molecules will occupy at least half of the available base sequences of mitochondrial DNA in an animal cell or one-quarter in fungal cells. Although the residual information content of mitochondrial DNA is limited, it may code for quite a number of proteins. It has been inferred (Schatz, 1970) that mitochondrial DNA codes for the protein which confers oligomycin sensitivity to ATPase, and for certain component(s) which confer erythromycin resistance on mitochondrial ribosomes (Linnane *et al.*, 1968b). There has so far been no direct experimental demonstration that this is so. Also, the mitochondrial genome has recently been implicated in coding for structural protein (Munkres and Woodward, 1966), cytochrome *c* oxidase (Edwards and Woodward, 1969), and even extramitochondrial membrane proteins (Attardi and Attardi, 1968).

Studies on the sites of synthesis of mitochondrial components *in vivo* have been facilitated by discoveries that chloramphenicol and erythromycin selectively inhibit activity of the mitochondrial protein synthesising system and cycloheximide that of the cytoplasmic protein synthesising system. These drugs are frequently used in studies with yeasts and *Neurospora* because of the ease with which they can be supplied to cultures of these organisms. In whole animals, problems of detoxification and inactivation of drugs have complicated the treatment but these have in some cases been overcome by ingenious experimental devices (Kroon and De Vries, 1969).

Eucaryotes such as yeasts and *Neurospora* cannot grow in the presence of cycloheximide, because general protein synthesis is inhibited. Therefore, experiments involving cycloheximide are restricted to short-term treatments since otherwise results become complicated by inhibition of cell

growth. On the other hand, eucaryotes especially yeasts, can grow apparently indefinitely in the presence of chloramphenicol or erythromycin. Any inhibitory effect of chloramphenicol on the synthesis of particular sub-cellular components will be readily observed during growth of the cell.

When a culture of yeast is propagated in the presence of chloramphenicol or erythromycin for many generations, these cells retain an active mitochondrial protein synthesising system (Davey, Yu and Linnane, 1969). From this observation, it has been inferred that the replication or multiplication of the mitochondrial genetic apparatus, such as mitochondrial DNA-polymerase, RNA-polymerase, mitochondrial ribosomal proteins and other enzymes involved in the activity of the mitochondrial protein synthesising system do not require active participation of the mitochondrial protein synthesising system. In other words, the mitochondrial genetic apparatus does not contain protein products of the mitochondrial protein synthesising system. This conclusion has been criticised on the basis that chloramphenicol and erythromycin do not suppress completely *in vitro* mitochondrial protein synthesis. This criticism is relevant in general to the evaluation of experimental results obtained with inhibitors *in vivo*. However, inhibition by chloramphenicol and erythromycin in yeasts is complete as assessed by the development of cell respiration, synthesis of cytochromes aa_3 and *b* and the ability to grow on nonfermentable substrates. Furthermore it is not certain whether incomplete inhibition of mitochondrial protein synthesis *in vitro* really reflects intrinsic insensitivity or isolation artefacts. *In vivo* assessment of mitochondrial protein synthesis indicated that chloramphenicol and erythromycin were within experimental error completely effective. That the mitochondrial DNA polymerase and RNA polymerase could not be translational products of the mitochondrial protein synthesising system is substantiated by recent results obtained with petites (Perlman and Mahler, 1970; Wintersberger, 1970). That mitochondrial ribosomal proteins

are synthesized by the cytoplasmic protein synthesising system was also substantiated by results of Kuntzel (1969) and Neupert *et al.* (1969).

A more significant conclusion that could be drawn from studies using chloramphenicol and erythromycin was that the synthesis and assembly of components of mitochondrial membrane (outer, inner and cristae) apparently does not require participation of the MPSS (see chapter 2; also Yu, 1968). Recent results from many laboratories indicate that soluble proteins of the mitochondrial matrix, (malic dehydrogenase and fumarase of the TCA cycle), proteins of the outer membrane and many proteins of the inner membrane (ATPase, ferrochelatase, ALA-synthetase, cytochrome *c*, etc.) are synthesized on the cytoplasmic ribosomes (see review by Schatz, 1970; Ashwell and Work 1970). In conjunction with a study on vegetative petites, it may be inferred that the genetic origin of these mitochondrial components is nuclear.

In summary, our present state of knowledge indicates that much is known about what the mitochondrial protein synthesising system does *not* synthesize and what mitochondrial genome does *not* code for, but practically nothing is known about what proteins it does synthesize and what protein(s) the mitochondrial genome does code for.

(iii) *In vivo* synthesis of cytochromes aa_3 and *b*

One of the most interesting problems of mitochondrial differentiation is the control of synthesis of the particulate cytochromes (i.e. cytochromes aa_3, *b* and c_1). As discussed in the last section unlike many other mitochondrial protein components which are known to be synthesized in the CPSS even when the mitochondrial system is inactive, the synthesis of these cytochromes apparently requires the active participation of both the MPSS and CPSS (Kraml and Mahler, 1967; Yu *et al.*, 1968; Henson *et al.*, 1968; Mahler *et al.*, 1968; Chen and Charalampous, 1969; Tuppy and Birkmayer, 1969). It has so far not been possible to obtain substantial and unilateral synthesis of cytochromes aa_3 and *b* when either of the two protein synthesising systems is inactive. Many hypotheses have been built on these rather negative results, and in general

they are variations of the experimental deduction that there is an interlock between the activities of the MPSS and the CPSS in the synthesis of cytochromes aa_3 and *b* (for review see Ashwell and Work, 1970).

In this thesis I have demonstrated that in *Candida parapsilosis* cytochromes aa_3 and *b* like many other protein components of mitochondria are synthesized in the CPSS. Unlike other mitochondrial proteins, the synthesis of these cytochromes is coupled or coordinated with the cellular potential for accelerated growth. Coordination between synthesis of cytochromes and rate of cellular metabolism might be mediated through a suppressor synthesized by the MPSS which counteracts the repressive control exerted by the cell on the synthesis of cytochromes. From the results of the inhibitor study using ethidium bromide, the genetic information for the synthesis of cytochromes aa_3 and *b* may be tentatively located in the mitochondrial genome. My hypothesis for the mechanism of synthesis of cytochromes in the cell has been described in detail in chapter 3 and will not be reiterated here. Instead I propose to speculate on the generality of the idea.

Up to the present time, the genetic origin of cytochromes aa_3 and *b* remains an open question (see review by Ashwell and Work, 1970). It is generally believed that structural genes for cytochromes aa_3 and *b*, like those for cytochrome *c*, are in the nucleus. This belief is based on the limited genetic information of the mitochondrial genome, and on recent reports by Tuppy and Birkmayer (1969) and Kraml and Mahler (1967) that apoprotein of cytochrome oxidase can be detected by immunological techniques in vegetative petites which apparently have lost normal mitochondrial DNA. However, the validity of the conclusion depends on the reliability of the immunological techniques used, in particular the selectivity or specificity of antibodies, in view of the fact that cytochrome oxidase (i.e. cytochromes aa_3) is an enzyme complex. Furthermore, although the genetic information of mitochondria may be limited, enough information is available to code for a substantial number of proteins of moderate size, especially in *Neurospora* and

yeast (Kuntzel, 1971). The pleiotropic nature of the petite mutation in yeast, the irreversible nature of this mutation and the absence of segregation of nuclear genes individually affecting synthesis of cytochromes aa_3, b and c_1 argue against the likelihood that structural genes of cytochromes aa_3, b and c_1 are in the nucleus. My allocation of the structural genes for cytochromes aa_3 and b to the mitochondrial genome is based solely on experimental results obtained with ethidium bromide. Ethidium bromide has been shown to inhibit selectively the transcription of the mitochondrial genome in HeLa cells (Zylber *et al.*, 1969). In my experimental systems, ethidium bromide inhibited the synthesis of mitochondrial RNA, M_1 and M_2 as well as cytochromes aa_3 and b, but not that of cytoplasmic ribosomal RNA (C_1 and C_2) and cytochrome c.

It has been suggested that ethidium bromide reacts also with other nucleic acid components (Kroon and de Vries 1971; and Waring, 1970 for detailed discussion) thus inhibiting not only transcription but also translation and replication. Under experimental conditions in which synthesis of cytochromes aa_3 and b, like that of cytochrome c, was no longer dependent on activity of the MPSS (i.e. in stationary phase cells of a CAP-treated culture), synthesis of cytochromes aa_3 and b, but not that of cytochrome c, was inhibited by ethidium bromide. This demonstrates that ethidium bromide cannot be held to inhibit the synthesis of cytochromes aa_3 and b at the level of translation in the CPSS, nor could ethidium bromide inhibit at the level of transcription of the nuclear genes because synthesis of cytochrome c (and of cytoplasmic ribosomal RNA) remains unaffected. It could be inferred from the present state of knowledge of the mitochondrial genome in yeasts that this is the most likely site of action of ethidium bromide. Thus, the correctness of the assignment of mitochondrial genome coding for cytochromes aa_3 and b rests on the validity of the idea that ethidium bromide is a specific inhibitor of mitochondrial transcription.

I have inferred a regulatory role for product(s) of the MPSS. However this does not exclude the possibility that

the MPSS may actually synthesize other protein(s) of catalytic or structural function. A regulatory role for the mitochondrion may also be inferred from many reported observations. In the following discussion, it is assumed that the mitochondrial genome contains structural genes for cytochromes aa_3 and b as well as a regulatory gene coding for a suppressor.

The "poky" mutant of *Neurospora* is a maternally-inherited mutant analogous to vegetative petites in yeast. It differs from petites in one characteristic way. Poky is respiratory-incompetent and lacks cytochromes aa_3 and b in the actively-dividing phase of growth i.e. in logarithmic phase; but it becomes respiratory-competent and synthesizes cytochromes aa_3 and b at stationary phase. The structural genes for the synthesis of cytochromes aa_3 and b thus cannot have been mutated. This phasic phenomenon in poky could be explained by the hypothesis outlined in chapter 3. In the case of poky, the cytoplasmic mutation may involve mutation of the suppressor gene in the mitochondrial genome. Thus, the suppressor may become ineffective in the suppression of the repressive control over transcription of the mitochondrial genome, when cells are engaged in active growth at logarithmic phase. When cultures of poky cease active growth, or division, repression of mitochondrial transcription for the synthesis of cytochromes aa_3 and b diminishes and synthesis of cytochromes occurs in spite of the defective suppressor.

The regulatory or suppressor role of the mitochondrial function can be used to explain the observation of cytoplasmic transformation reported by Diacumakos *et al.* (1965). They injected mitochondria from cytoplasmic mutants of *Neurospora* abn-1 and abn-2, into wild type cells. Characteristics of abn-1 and abn-2 mutants were transmitted to recipient cells resulting in a production of respiratory-deficient mitochondria. Diacumakos' experiment was in essence analogous to classical cis-trans tests for regulator and operator in bacterial genetics. In these maternally-inherited mutants of *Neurospora*, the suppressor gene in the mitochondrial genome might be mutated to become another

repressor instead of a suppressor. When these mutant mitochondria are introduced into the cytoplasm of wild-type cells, the mutant suppressors (which might act now as some form of repressor) could continue to be synthesised and could act on the transcription of the mitochondrial genome of wild-type mitochondria in recipient cells. These mutant characteristics would be perpetuated in recipient cells as long as mutant mitochondria were multiplied alongside normal, wild-type mitochondria.

Many "unstable" respiratory-deficient mutants have been described for obligate aerobes such as *Schizosaccharomyces pombe* (Heslot *et al.*, 1970 b) which show similar phasic phenomena as poky mutants of *Neurospora*. These mutants cannot be mutants defective in the structural genes for cytochromes aa_3 and b because these enzymes are synthesized late in the growth cycle. It appears more likely that they are mutants defective in the regulation of synthesis of cytochromes in the manner I have described.

The possible role of mitochondria in the production of a diffusible or mobile suppressor molecule communicating with the nucleus and cytoplasm can be envisaged for the phenomenon of suppressiveness in petite mutants of *Saccharomyces*. Cytoplasmically-inherited petite mutants of *Saccharomyces* are classified as neutral or suppressive (for review, see Wilkie, 1966). Neutral haploid petites are those which when crossed with a normal haploid grande, give rise to normal grande diploids on vegetative growth. Suppressive haploid petites are those which when crossed with a normal haploid grande, give rise to a mixture of normal grandes and respiratory deficient diploids on vegetative growth. The proportion of grande and respiratory-deficient diploids varies according to the degree of suppressiveness of the haploid petite. The degree of suppressiveness ranges from zero (for neutral petites) to over 90%. Diploidization of a normal grande and an isogenic suppressive petite is analogous to Diacumakos' experiment of cytoplasmic transformation in *Neurospora*. By introducing the aberrant genetic apparatus of mutant mitochondria (by

the fusion of two isogenic haploids to form a diploid), expression of the genetic apparatus in normal mitochondria is interrupted hence transcription of genes for cytochromes aa_3 and *b*, resulting in the formation of a respiratory-deficient diploid. Thus it may be envisaged that mitochondria of suppressive petites produce some heritable form of mutant suppressor molecules which instead of counteracting or inactivating, accentuate or exaggerate the repressive control on the transcription of mitochondrial genome in grande mitochondria thus resulting in a complete cessation of mitochondrial transcription and respiratory deficiency.

A mutant of *Saccharomyces* has been described by Wilkie which shows changes of respiratory function with the cultural environment. This mutant called *gi* (glucose-induced) is a nuclear-gene mutant which in the presence and absence of glucose gives rise to vegetative petite and grande progeny respectively. To explain this phenomenon, a repressive control on the mitochondrial genome has been evoked (Roodyn and Wilkie, 1968).

It should be noted that wild-type *S. cerevisiae* (grande) shows no phasic response towards inhibition by chloramphenicol and erythromycin on the synthesis of cytochromes. This does not exclude the possibility of some form of interaction between the mitochondrial genetic apparatus and the nuclear genetic apparatus. Thus, the linkage of repression to cellular growth and division in *Candida* may not apply in *Saccharomyces*.

The possible regulatory role of the mitochondrial genetic apparatus in cellular synthesis of cytochromes aa_3 and *b* (respiratory enzymes) is not new. Ephrussi (1953) has suggested that two genetic determinants, nuclear and cytoplasmic (mitochondrial) are related. In his view, the synthesis of cytochromes aa_3 and *b* is directly controlled by the cytoplasmic determinant as seen in the case of cytoplasmic petites. However, the function and expression of the cytoplasmic determinant is governed by a higher control ------ nuclear genes. Only when nuclear genes are in the normal and dominant state is the cytoplasmic

determinant functional. Thus, the respiratory-competent wild-type (grande) yeast requires the simultaneous presence of normal dominant nuclear genes and the cytoplasmic determinant. The conclusion reached in this thesis is therefore in agreement with Ephrussi's hypothesis put forward 20 years ago.

6-1-2 MITOCHONDRIAL RIBOSOMES

It is beyond the scope of this thesis to discuss critically the very extensive literature now available concerning mitochondrial ribosomes and RNA. This has been done very effectively in a number of recent reviews (Schatz, 1970; Ashwell and Work, 1970; Borst and Grivell, 1971; Kuntzel, 1971). From these at least two things are clear. Firstly, there is no single universal value for the size of mitochondrial ribosomes and secondly, there is still some dispute as to the size of these particles within a given organism. Much of the apparent disagreement about size may be due to technical factors such as precise conditions of sedimentation and the validity of the sedimentation value of the reference marker, and hence may not necessarily reflect differences in intrinsic particle sizes. But this does not account for all the differences in the literature, and especially alleged differences between sizes of mitochondrial ribosomes of mammals and lower eucaryotes. To assess how valid is this generalization, I propose to discuss some key reports in this field which have strongly influenced the current view on mitochondrial ribosomes. The general view is that mitochondrial ribosomes from vertebrates have a size generally in the range 50-60*s* (called by some, miniribosomes); those of fungi (or lower eucaryotes) are said to be about 70-80*s*.

Some assessment of the purity of mitochondrial preparations is of particular relevance because the extent of initial contamination with cytoplasmic ribosomes will determine to what extent other criteria need be used to identify mitochondrial ribosomes among the contaminants.

(i) Mitochondrial ribosomes of fungi (Yeast and *Neurospora)*

A few criteria have been employed for assessing purity of mitochondrial preparations. One assessment of contamination uses highly-labelled radio-active cytoplasmic ribosomes. The procedure is to add radio-active microsomes to a cell-free homogenate before isolation of mitochondria and to assess the level of contamination from the residual radio-activity in the purified mitochondrial fraction. Schmitt (1969) working with *S. cerevisiae* reported that ribosomes extracted from the mitochondrial fraction, estimated from studies using radio-active cytoplasmic ribosomes to be contaminated by less than 3% and constant in specific activity of cytochrome *c* oxidase, had a sedimentation value of 80*s* and could not be physically separated from cytoplasmic ribosomes. However, these 80*s* particles extracted from purified mitochondrial fractions were different from cytoplasmic ribosomes in terms of dissociation by high concentrations of KCl. Recently, Schmitt (1970) by extensive washing of isolated mitochondria with EDTA-containing buffer and using buffer of high ionic strength during extraction of ribosomes was able to extract 72*s* particles from the purified mitochondrial preparation of *S. cerevisiae*. The 72*s* particles were regarded as mitochondrial ribosomes, and the 80*s* particles which he had previously extracted from the same purified mitochondrial fraction were mitochondrial-specific, membrane-bound cytoplasmic ribosomes. From Schmitt's reports it is clear that (1) the criteria by which he assessed mitochondrial purity in the earlier report (Schmitt, 1969) were not absolutely reliable, and (2) depending on where they are localized, cytoplasmic ribosomes can have different physical properties, such as dissociation by KCl. The reliability of the reconstitution experiment in assessing degrees of purity of mitochondrial fractions is shown in Table 6-1. Based on the residual radio-activity in the mitochondrial fraction after 5 washes with buffer containing Mg^{++}, a value of about 20% contamination of mitochondria by cytoplasmic ribosomes was calculated. However when RNA was extracted from such preparations, very little high

SUBCELLULAR FRACTION	^{3}H-URACIL RECOVERED (cpm/mg protein)	(cpm/mg RNA)
WH	0	0
^{3}H-PMS	18000	149
MIXTURE	7000	63
^{3}H-SOL	8540	71
MT x3	1340	13
MT x5	1090	11

CONTAMINATION	IN TERMS OF PROTEIN	IN TERMS OF RNA
$\frac{\text{residual cpm in MT x5}}{\text{cpm in mixture}}$	15%	17.5%

	BASE COMPOSITION OF RNA IN SUBCELLULAR FRACTIONS mole percent				
	UMP	GMP	AMP	CMP	GMP+CMP
^{3}H-PMS	25.3	27.8	26.1	20.9	48.7
MIXTURE	27.1	27.2	25.3	20.4	47.6
^{3}H-SOL	27.9	28.9	23.3	20.0	48.9
MT x3	28.6	30.3	23.2	17.9	48.2
MT x5	29.6	25.6	27.2	17.7	43.3

Table 6-1. Estimation of contamination by cytoplasmic ribosomes.
Unlabelled cells were converted to protoplasts by snail-gut enzyme digestion as described in Section 3-2-2. Protoplasts were then lyzed by homogenization, and the lyzate differentially centrifuged to yield a cell-free homogenate (denoted WH). It should be noted that EDTA was present during these procedures. Highly labelled cytoplasmic ribosomes in the postmitochondrial supernatant was prepared in the following manner. Cells cultured in the presence of ^{3}H-uracil were harvested at late logarithmic phase. The labelled cells were broken by homogenization with glass beads in a MSK Braun shaker as described in Section 4-2-2. The brei was differentially centrifuged to yield a labelled-post-mitochondrial supernatant (denoted as ^{3}H-PMS). The whole process of obtaining ^{3}H-PMS was carried out in the presence of Mg++ (10 mM). The reconstitution experiment was carried out as follows. Equal volumes of unlabelled cell-free homogenate (WH) and labelled-postmitochondrial supernatant were mixed and the mixture allowed to equilibrate for 1 hr at 0°C. The mixture was then differentially centrifuged at 10000 rpm for 20 min (Sorvall, SS34) to sediment a crude mitochondrial fraction and a derived postmitochondrial supernatant (denoted as ^{3}H-SOL). The crude mitochondrial fraction was washed by resuspension and sedimentation with 20 volumes of sucrose AMT buffer (sucrose 0.5M, NH_4Cl 0.1M, $MgCl_2$ 0.01M, TrisHCl 0.01M pH 7.6). The 3-times and 5-times washed mitochondria are denoted as MT x3 and MT x5 respectively. Sub-

cellular fractions were processed for determination of radio-activities. RNA in the subcellular fractions were hydrolysed with KOH and analysed according to the method described by Katz and Coomb (1963).

molecular weight RNA other than that of cytoplasmic ribosomes was detected. Base composition analysis of the extracted RNA indicated that it was routinely 42-43% G+C, suggestive of over 60% contamination by cytoplasmic ribosomes.

There was thus a striking difference between contamination calculated from radio-activity data, and from chemical analysis. A possible explanation would be that mitochondria might already have been heavily contaminated with unlabelled cytoplasmic ribosomes or microsomes bound to mitochondrial membrane in cell-free homogenates and little exchange occurred when labelled cytoplasmic ribosomes were added and equilibrated.

Vignais *et al.* (1969) after careful checking for cytoplasmic ribosomal contamination by electron microscopy, showed that ribosomal particles extracted from purified mitochondrial fractions of *C. utilis* were 77-80s in size. After incubating mitochondria with labelled amino acid *in vitro* these particles were found to be labelled. They showed further that the *in vitro* labelling of the whole mitochondrial fraction with C^{14} amino acids was sensitive to inhibition by chloramphenicol and resistant to cycloheximide, but it was not demonstrated that the C^{14} labelling of extracted ribosomal particles showed similar differential sensitivity to chloramphenicol and cycloheximide. It is implicitly assumed by Vignais *et al.* (1969) that when a mitochondrial preparation exhibits characteristic mitochondrial amino acid incorporating activity it is free of contamination by cytoplasmic ribosomes. However it should be realized that the incubation system used for mitochondrial incorporation is unfavourable for cytoplasmic ribosomal incorporation because it lacks pH 5.1 enzymes, GTP, and added messenger RNA and therefore cytoplasmic ribosomes (when they are present) cannot be detected by this means. Therefore, the fact that an active mitochondrial preparation is sensitive to CAP and resistant to cycloheximide does not mean that it is uncontaminated.

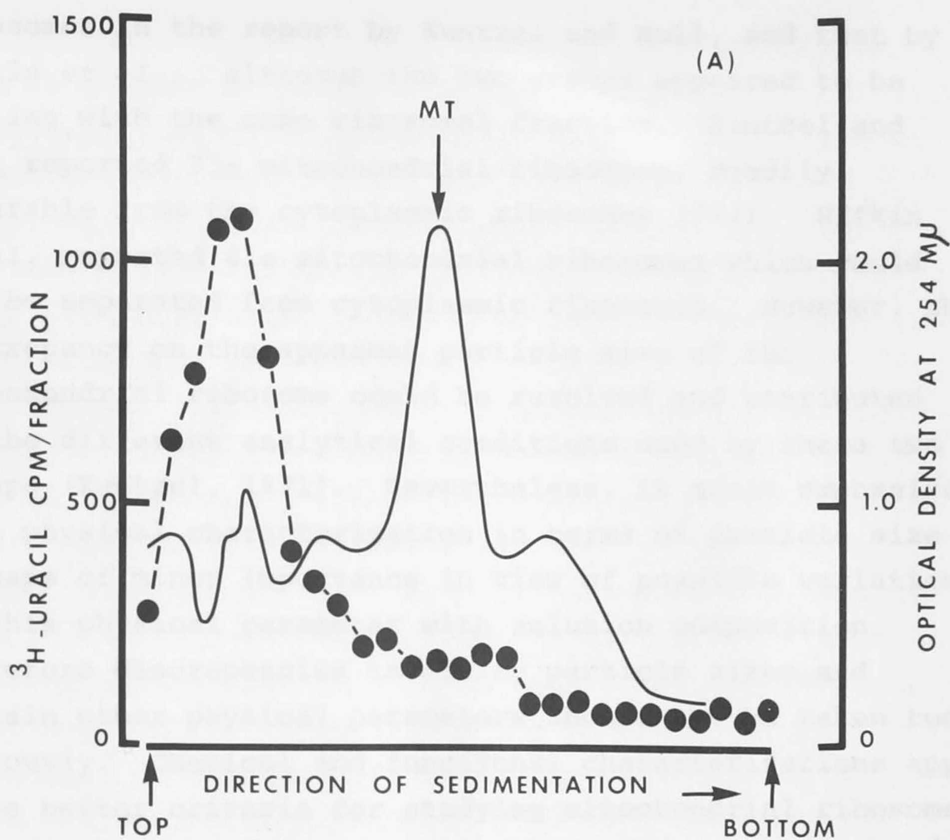
Enzyme markers such as glucose-6-phosphatase (Bartoov *et al.*, 1970) have been used to assess microsomal

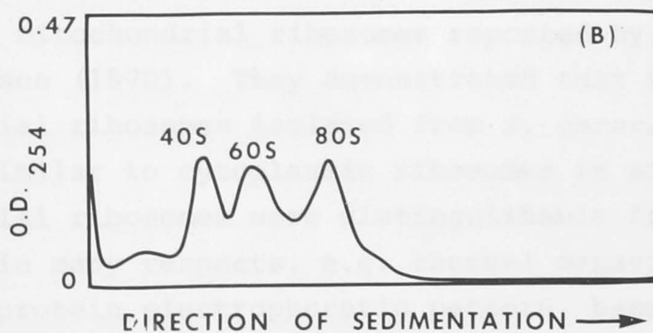
contamination of mitochondrial fractions in mammalian tissues. This criterion is generally more controversial and less rigorous than those just discussed. In lower eucaryotes such as *Neurospora* and yeasts, satisfactory microsomal markers are not known. Also procedures such as extensive washing to constant specific activity of cytochrome *c* oxidase (Bartoov *et al.*, 1970; Leon and Mahler, 1968) or some other mitochondrial enzyme markers, and isokinetic or equilibrium banding of mitochondria (Wintersberger, 1967) do not guarantee purity if cytoplasmic ribosomes remain adsorbed to mitochondrial membranes. Indeed as shown in Figure 6-1, isokinetic banding of mitochondria in either Mg^{++}-or EDTA-buffer appears to be no guarantee of elimination of cytoplasmic contamination.

In earlier studies, emphasis was placed on the physical characterization of mitochondrial ribosomes. Attempts were made to substantiate the concept that mitochondrial and chloroplastic ribosomes are 70*s* like bacterial ribosomes. Physical characteristics similar to those exhibited by bacterial ribosomes were frequently looked for, because of certain similar sensitivities of mitochondrial and bacterial protein synthesis towards inhibitors (Wintersberger, 1967; Rogers *et al.*, 1967). It generally became obvious on reconsideration that functional similarities between mitochondrial and bacterial protein synthesis need not reflect physical and/or chemical similarities of the participating ribosomes. In this regard, it is worth considering the properties of mitochondrial ribosomes from *Neurospora crassa* described by Kuntzel and Noll (1967), and Rifkin, Wood and Luck (1967). These workers demonstrated that these mitochondrial ribosomes of *Neurospora crassa* were different from both cytoplasmic and bacterial ribosomes in terms of particle size and chemical constitution (RNA species and base composition) although mitochondrial and bacterial ribosomes shared certain physical properties such as dissociation in low concentrations of magnesium. There was disagreement on the particle size of the mitochondrial

Figure 6-1. Isokinetic banding of mitochondria and sedimentation analysis of ribosomal particles extracted from mitochondria.

Labelled postmitochondrial supernatant (H^3-PMS) and unlabelled cell-free homogenate (WH) were prepared according to procedures described in Table 6-1. A reconstitution of H^3-PMS and WH similar to that described in Table 6-1, was carried out and a crude mitochondrial fraction was sedimented from the resultant mixture. The crude mitochondrial fraction was washed three times, resuspended in STE (sorbitol 0.5M, Tris HCl 0.01M pH 7.4, sodium EDTA 0.5mM), layered over a continuous sucrose density gradient of 30-70% w/v sucrose in Tris HCl (0.01M pH 7.4)-EDTA (0.5mM) and centrifuged at 24000 rpm for 1 hr at 4°C (Spinco, SW 25.1). Mitochondria which banded at about 45% sucrose was identified by the peak of enzymic activities of cytochrome *c* oxidase and succinate-cytochrome *c* reductase. Distribution of radio-activity in the gradient was determined. (A): the peak of radio-activity did not penetrate far into the gradient and remained near the top of the gradient. The mitochondrial zone was removed and sedimented by differential centrifugation; and washed once again with mitochondrial resuspension buffer. Calculation of contamination by residual radio-activity indicated that it was less than 0.1% contaminated by cytoplasmic ribosomes. Ribosomal particles were extracted from the purified mitochondrial fraction with Triton X-100 as described in Section 4-2-6 and analyzed in continuous sucrose density gradients as described in Section 4-2-7. The peaks seen in Figure 6-1 (B) were identified as 80*s*, 60*s* and 40*s* by comparison with yeast cytoplasmic ribosomes and analytical ultracentrifugation in a Molel E Spinco. The base composition of the RNA in the ribosomal particles extracted from the purified mitochondrial fraction is shown in the Table insert of Figure 6-1.





BASE COMPOSITION OF RNA IN MITOCHONDRIAL RIBOSOMAL PREPARATION	MOLE %
U	29.0
G	24.9
A	28.0
C	18.1
G+C	43.0

ribosomes in the report by Kuntzel and Noll, and that by Rifkin *et al.*, although the two groups appeared to be dealing with the same ribosomal fraction. Kuntzel and Noll reported 73*s* mitochondrial ribosomes, readily separable from the cytoplasmic ribosomes (77*s*). Rifkin *et al.* reported 81*s* mitochondrial ribosomes which could not be separated from cytoplasmic ribosomes. However, the discrepancy on the apparent particle size of the mitochondrial ribosome could be resolved and attributed to the different analytical conditions used by these two groups (Kuntzel, 1971). Nevertheless, it again emphasizes that physical characterization in terms of particle size is perhaps of minor importance in view of possible variations of this physical parameter with solution composition. Therefore discrepancies involving particle sizes and certain other physical parameters should not be taken too seriously. Chemical and functional characterizations appear to be better criteria for studying mitochondrial ribosomes and this is well illustrated in the careful and detailed studies of mitochondrial ribosomes reported by Morimoto and Halvorson (1970). They demonstrated that although pure mitochondrial ribosomes isolated from *S. cerevisiae* were 80*s*, and thus similar to cytoplasmic ribosomes in size, mitochondrial ribosomes were distinguishable from cytoplasmic ribosomes in many respects, e.g. thermal denaturation, ribosomal protein electrophoretic pattern, base composition and behaviour in gel electrophoresis of RNA constituents. Besides these physical and chemical criteria, their mitochondrial ribosomes were distinguishable from cytoplasmic ribosomes in that they could be reconstituted to support amino acid incorporation into polypeptides in a system which was sensitive to CAP and resistant to CHI (Scragg *et al.*, 1971).

(ii) Mitochondrial ribosomes of vertebrates

Biochemical studies of mitochondrial ribosomes in mammalian tissue were first described some years ago with reports (Truman, 1963; Elaev, 1964) that mitochondrial fractions of rat liver contained ribosomes which were physically indistinguishable from cytoplasmic ribosomes.

O'Brien and Kalf (1967) were critical of these earlier results because no chemical or functional tests of their authenticity as mitochondrial ribosomes were described, and no precautions to guard against contamination by cytoplasmic ribosomes or microsomes were taken. In a detailed study O'Brien and Kalf confirmed Truman's and Elaev's results using standard methods for the preparation of mitochondria from rat liver. However, they were able to eliminate 78*s* ribosomes by repeated washing of the mitochondrial fraction with Mg^{++}-free buffer and obtained, instead an extremely low yield of 55*s* particles. Kalf and O'Brien argued that 78*s* particles were cytoplasmic contaminants because (1) 78*s* particles were not labelled with C^{14}-amino acid when mitochondrial fraction washed six times were incubated with radio-active amino acid *in vitro*, (2) the yield of 78*s* particles was inversely proportional to the number of washes, and (3) electron microscopy of positively-fixed mitochondrial fractions showed that ribonucleoprotein particles inside were smaller than those outside. Their arguments that the 55*s* particles are mitochondrial ribosomes were as follows: The mitochondrial fraction washed extensively without magnesium ions was low in RNA content (10 μg/mg protein), and contained one free ribosome (cytoplasmic) per 5 mitochondria by electron-microscopic observation. Sucrose density gradient centrifugation of the ribosomal preparation extracted from washed mitochondria revealed no 78*s* peak but only a 55*s* peak, and this became labelled with C^{14}- amino acid when mitochondria were incubated *in vitro*. It should be noted that although these workers showed that repeated washing of rat liver mitochondria eliminated the 78*s* particles, they did not present any evidence of identity of the 78*s* particles and cytoplasmic ribosomes. During repeated washing of rat liver mitochondria with Mg^{++}-free buffer, Kalf and O'Brien did not observe a progressive increase or enrichment of the 55*s* particle, which would suggest that it might be a derivative of the 78*s* particle when Mg^{++} ion concentration was diluted, by washing, below a critical level. The fact that a particle can be eliminated from a larger

structure such as the mitochondrion by repeated washing does not necessarily mean that its origin is not from within the structure itself. Also, the fact that 55*s* particles were labelled with C^{14} amino acid when mitochondria were incubated *in vitro* does not constitute a functional characterization of these particles because they were not shown to respond differentially to inhibition by specific inhibitors of protein synthesis. Also, it has not been demonstrated that 60*s* subunits of the cytoplasmic ribosomes incubated under conditions that favoured cytoplasmic ribosomal incorporation would be labelled. Although Kalf and O'Brien's experiments constituted a thorough and critical study of the problem of contamination, the characterization of their 55*s* particles is not satisfactory in a number of respects. Nevertheless, the practice of extensive washing and purification by gradient centrifugation in Mg^{++}-free buffer or EDTA-containing buffer has since been advocated by many workers as a means of eliminating contamination by cytoplasmic ribosomes (Leon and Mahler, 1968; Bartoov *et al.*, 1970; Vesco and Penman, 1969). However, if this procedure is adopted it needs to be established firstly, whether or not mitochondrial ribosomes within mitochondria became dissociated as well as contaminating cytoplasmic ribosomes when mitochondrial fractions are washed with EDTA or Mg^{++}-free buffers and secondly, whether or not mitochondrial ribosomal subunits once dissociated will reassociate to form bipartite ribosomes when Mg^{++} is added during subsequent extraction of ribosomes from the organelle.

Recently, Swanson and Dawid (1970) reported that the mitochondrial ribosome of frog oocytes was a 60*s* particle. The 60*s* particle contained two species of high molecular weight RNA which were hybridizable only with mitochondrial DNA of frog oocytes. Furthermore, the 60*s* particle was active in phenylalanine incorporation into polypeptide using polyuridylic acid as messenger. They postulated that the 60*s* particle may be found in all mitochondria that contain mitochondrial DNA circles of 5 µm (i.e. vertebrate or mammalian mitochondria). Yeast and *Neurospora* mito-

chondria contain larger DNA molecules and have 70*s*-80*s* type ribosomes. In order to substantiate this hypothesis, it is most important to show that the 60*s* particle is in fact a ribosome of bipartite structure. There are three criteria that need to be satisfied to identify a particle as a ribosome in the classical sense. Firstly, it ought to be a ribonucleoprotein capable of dissociation in low Mg^{++} concentration. Secondly, it must be a macromolecular assembly of protein and RNA subunits and lastly, it must be able to participate in protein synthesis which shows characteristic inhibitor responses. Although Swanson and Dawid showed the presence of 43*s* and 32*s* particles in conjunction with a 60*s* particle in the same sucrose density gradient on centrifugation of a ribosomal extract of frog oocyte mitochondria, this was no proof that the 60*s* particle is composed of a 43*s* and a 32*s* particle. It remained to be demonstrated that the 60*s* particle can be dissociated in low Mg^{++} concentration into subunits (43*s* and 32*s* particles). The strongest argument in favour of the 60*s* particle being a ribosome was the presence of two high molecular weight RNA species. However, these might result from scission of the RNA polynucleotide chain in the larger subunit of a mitochondrial ribosome resulting in the appearance of two large fragments. This type of specific scission of the RNA chain is not uncommon, and is frequently observed in chloroplastic ribosomal RNA (Ingle *et al.*, 1970) and in cytoplasmic ribosomal RNA of many insects (Applebaum *et al.*, 1966; Greenberg, 1969). The RNA of the 60*s* particle of Swanson and Dawid is likely to be distinct from that of cytoplasmic ribosomes because of its specific hybridization to mitochondrial DNA. As a corollary, it is likely that the 60*s* particle was an authentic mitochondrial ribosomal particle though it might only be a subunit of the original ribosome.

The fact that the 60*s* particle could support the incorporation of phenylalanine into TCA-insoluble material in the presence of polyU does not necessarily qualify it as a ribosome because it has not been demonstrated that

this incorporation represented polypeptide synthesis sensitive to inhibitors of the MPSS and insensitive to inhibitors of the CPSS.

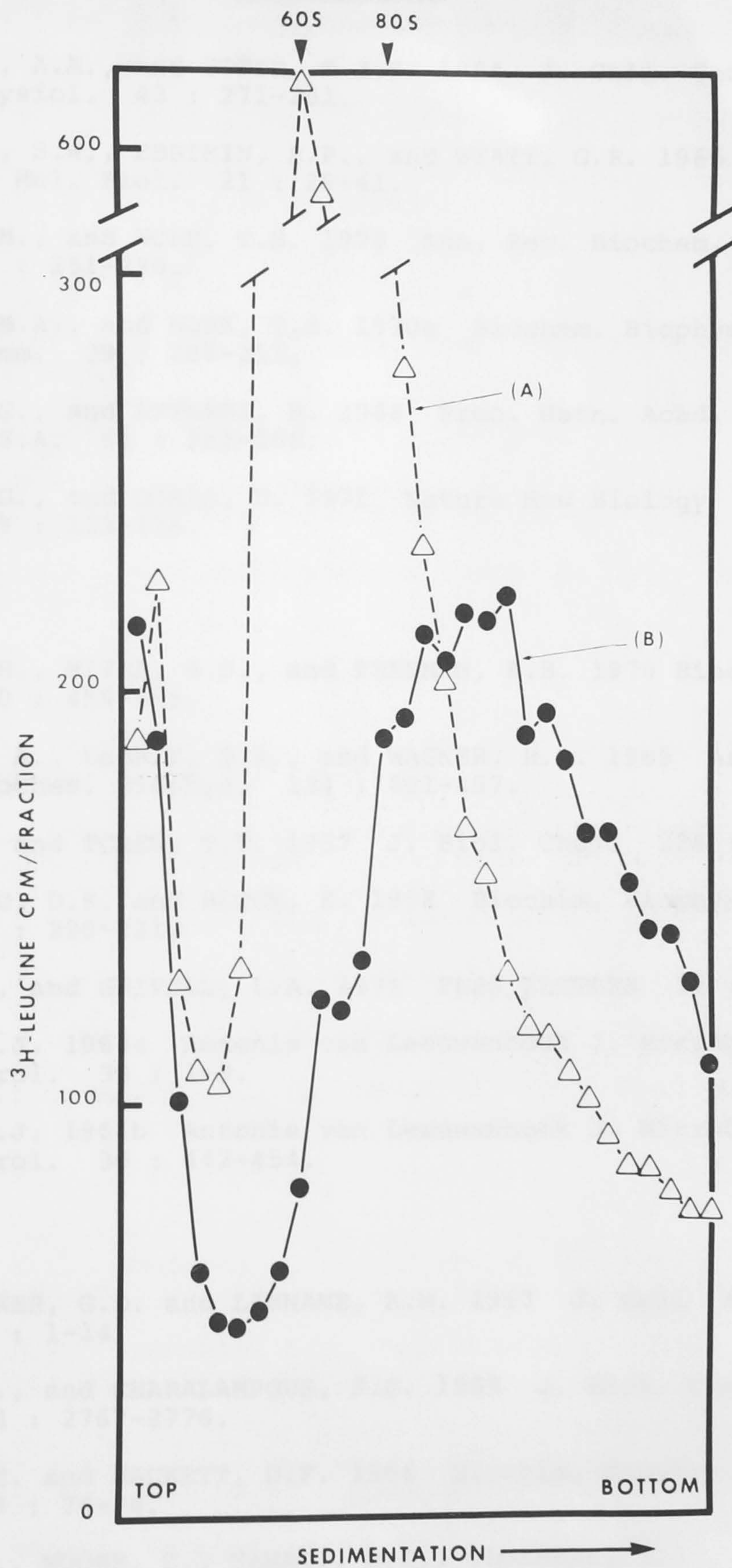
Functional characterization of ribosomes has recently been gaining support as a means of identifying ribosomes in mixtures. Two recent reports (Ashwell and Work, 1970 a; Attardi and Ojala, 1971) demonstrate amino acid incorporation (or binding) by 60*s* particles from mitochondria from rat liver and HeLa cells sensitive towards chloramphenicol and resistant to cycloheximide. However, 60*s* particles reported by Ashwell and Work, and Attardi and Ojala were not demonstrated to be bipartite structures. It should be pointed out that in the report by Attardi and Ojala, 74*s* particles were observed in addition to the 60*s*, 45*s* and 35*s* particles in the ribosomal extract of mitochondrial fractions when they were analyzed on sucrose gradients. The 74*s* particles were regarded as cytoplasmic ribosomal contaminants. It is significant that when the functional characterization was carried out, the 74*s* particle was labelled in the presence of cycloheximide, and this cycloheximide-resistant labelling was sensitive to both puromycin and chloramphenicol. Thus the 74*s* particle behaved like the 60*s* particle in a functional test in a manner expected of mitochondrial ribosomes. Attardi and Ojala assumed the 74*s* particles, which they conveniently regarded as cytoplasmic ribosomal contaminants in the early section of their report, to be dimers of the 60*s* particles responding similarly to them in a functional test. It is very unlikely that an ellipsoidal particle of twice the mass of a 60*s* particle would have a sedimentation constant of less than 80*s*. It would seem more likely that the 74*s* particles were authentic mitochondrial ribosomes of bipartite structure and the 60*s* particles were derivatives or subunits of them. The fact that 60*s* particles became labelled when HeLa cells were pulsed with radio-active amino acid is not surprising. Perlman and Penman (1970) have demonstrated that radio-active amino acid label could remain attached to particles derived from mitochondrial ribosomal particles (95*s*) when

the latter were dissociated in the presence of EDTA. A result similar to that of Perlman and Penman was obtained when cycloheximide-resistant labelling of mitochondrial ribosomes in *S. cerevisiae* was dissociated in EDTA (Fig. 6-2).

While studies of Swanson and Dawid, Ashwell and Work, and Attardi and Ojala left little doubt that their 60*s* particles were mitochondrial and ribosomal in nature, it remains to substantiate that the 60*s* particle is really a ribosome of bipartite structure. Indeed, this uncertainty was expressed by Ashwell and Work, who were unconvinced that the 60*s* particle of their study represents the monomeric form of the ribosome rather than the large subunit.

In summary, the hypothesis that mammalian mitochondrial ribosomes are smaller than fungal mitochondrial ribosomes because mitochondrial genomes of mammalian cells are generally smaller than those of fungi is an interesting hypothesis with evolutionary overtones. The experimental evidence for a mitochondrial miniribosome in mammalian cells is in general circumstantial and as yet inconclusive.

Figure 6-2. Effect of EDTA on the distribution of radio-activity in mitochondrial ribosomes. Ribosomal particles extracted from mitochondrial fraction of cells which had been pulse-labelled with ^{3}H-leucine in the presence of cycloheximide (100 μg/ml) as described in Section 5-2-2, were analyzed in sucrose density gradients containing either magnesium ion (10 mM) or EDTA (1 mM) in Tris HCl (10 mM) pH 7.4 and NH_4Cl (10 mM) for distribution of radio-activity. Radio-activity in gradients containing magnesium was associated with the 80*s* and ribosomal aggregate regions of the gradient (curve (B)). In the presence of EDTA, the labelling was shifted to 60*s* region of the gradient (curve (A)).



60S
80S
600
300
200
100
0
3H-LEUCINE CPM/FRACTION
(A)
(B)
TOP
BOTTOM
SEDIMENTATION

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