The Genetics of Glucosamine Resistance in Yeast

J. J. Elliot B.Sc.

Department of Biological Sciences

(Submitted in partial fulfillment of
the requirements for the degree of
Master of Science)

B R O C K U N I V E R S I T Y

St. Catharines, Ontario, L2S 3A1

August 1975

Abstract

A preliminary genetic analysis was carried out on 14 mutants of Saccharomyces cerevisiae resistant to respiratory repression induced by D(+) glucosamine. One mutant strain (GR133) carried a nuclear mutation (designated gay-1) conferring glucosamine resistance. Five strains (GR7, GR62, GR120, GR124 and GR125) were either sterile or carried conditional lethality precluding further genetic analysis. The remaining group of mutants (GR5, GR6, GR8, GR9, GR10, GR22, GR112 and GR127) were found to carry one or more cytoplasmic G^R determinants.

Detailed investigation of GR10 and GR6 derivatives demonstrated that:

1. G^R can be reduced or eliminated by ethidium bromide (EB) treatment.
2. G^R can be quantitatively enriched in glucosamine containing medium.
3. a cross between a G^R enriched GR10 derivative and a neutral EB induced petite displayed phenotypic G^R dominance.
4. GR6 and GR10 derivatives were sensitive to oligomycin, erythromycin and chloramphenicol.
5. stock strains resistant to these drugs were glucosamine sensitive.

These observations 1 →5 suggest that GR10 and probably GR6 carry novel mutation(s) located on mitochondrial DNA. The locus of GR10 was designated [CAT-1].

Acknowledgments

I would like to thank Dr. A. J. S. Ball for whenever possible, guiding this work, rather than leading it. The resultant contribution to my problem solving skills has already proven invaluable.

I would also like to thank my wife Kathryn for constant encouragement and Mary Butryn for her patience and industry in typing this manuscript.

Table of Contents

List of Tables

List of Illustrations

(Figures and Plates)

Nam et ipsa scientia potestas est.

Knowledge itself is power.

FRANCIS BACON:

from Religious Meditations of Heresies.

I N T R O D U C T I O N

A great deal of interest exists today in mitochondrial function and biogenesis. The appearance of several volumes dealing exclusively with this area in recent years (ERNSTER and DRAHOTA 1969, ROODYN and WILKIE, 1970, MILLER 1970, BOARDMAN SMILLIE and LINNANE 1971, KROONE and SACCONE 1974) bears testimony to the growth of interest in mitochondrial biogenesis within the past 10-15 years. The task of elucidating mitochondrial functions has been undertaken with the tools of biochemistry, molecular biology and genetics largely with the co-operation of a facultative yeast, Saccharomyces cerevisiae. Interest has been very strong in characterization of mitochondrial gene products and in clarification of the relationship between mitochondrial and cytosolic protein synthesis (reviewed by SHATZ and MASON 1974).

Towards this end, the groups of Slonimski and Linnane have made great strides in establishing yeast mitochondrial genetics as a dynamic field and as a probe for the genetic mechanisms involved in control of mitochondriogenesis. However, much research still lies ahead before these problems can be considered solved.

Most accomplishments to date in the isolation of mitochondrial mutants have been concerned with "structural" mutations (eg. respiratory chain lesions). It is our contention that not until control function mutations have been isolated and characterized, can an integrated understanding of the genetic control mechanisms involved in mitochondriogenesis be gained (much as occurred with the bacterial operon theory, JACOB and MONOD 1961; PERLMAN, CHEN, DE CROMBRUGGHE, EMMER, GOTTESMAN, VARMUS and PASTAN, 1970)

It was felt that a phenomenon termed the Crabtree effect (also glucose effect or reverse Pasteur effect, for review see IBSEN 1961) offered the greatest potential for investigating mitochondrial control phenomena. The Crabtree effect consists essentially of the repression of mitochondrial functions (ie. respiration) via the stimulatory effect on fermentation of excess fermentable substrates (eg. glucose). The drawback to simple genetic analysis of this response is its transitory nature (ie. when glucose is exhausted respiration becomes derepressed (IBSEN 1961). This was overcome by the utilization of a gratuitous respiratory repressor, D (+) glucosamine, which mimics glucose induced catabolic repression (Crabtree effect, LETANSKY 1968) but irreversibly, allowing the use of conventional microbiological methods (plating on differential media) for mutant analysis. Mutants (U.V. induced) resistant to glucosamine-induced catabolite repression were isolated in the laboratory of Dr. A. J. S. Ball and preliminary descriptions have been published (ELLIOT and BALL 1973, ELLIOT and BALL 1974, ERRINGTON and BALL 1974, ELLIOT and BALL 1975)

The present study was undertaken to characterize the inheritance pattern of selected glucosamine resistant mutants in the hope that this would give some insight into the probable localization(s) of the molecular lesion(s) which bestow glucosamine resistance on these isolates.

CHAPTER ONE

REVIEW OF THE LITERATURE

A. The Crabtree Effect: Respiratory Catabolite Repression

The ability of solid tumors to demonstrate respiratory inhibition and "higher than normal" glycolytic activity upon addition of glucose was first described by CRABTREE (1929) in slices of solid sarcomas and carcinomas. This phenomenon has been called the Crabtree effect, glucose effect and reversed or inverted Pasteur effect. Coining of the latter designation was due to this effect (inhibition of respiration by glycolysis) being the inverse of the phenomenon described by Pasteur, the inhibition of glycolysis by respiration under O_2 stimulation, called the Pasteur effect. In addition to many neoplastic cell lines, the Crabtree effect has been described in a number of cell types derived from normal human and mammalian tissues (for review see IBSEN 1961). It was also described by BELITZER (1936) in the faculatative yeast Saccharomyces cerevisiae. Belitzer also suggested that competition between glycolysis and respiration for common intermediates might be the basis of the Crabtree effect.

A theory that did not enjoy much popularity was that the Crabtree effect was due to a respiration inhibiting decrease in pH due to H+ release during glycolysis. This was based on the observation that respiration was pH dependent (TIEDEMAN 1952). EMMELOT and BOS (1959) however, pointed out that the pH inhibition theory did not explain the ability of a non-glycolysed glucose analogue, deoxyglucose, to elicit a Crabtree effect in ascites cells. The theory of BELITZER remained the most popular.

Support for BELITZER'S theory and the suggestion that inorganic phosphate (Pi) and/or adenine nucleotides were the competed for intermediates came from the demonstration that uncoupling of phosphorylation from

oxidation by dinitrophenol (DNP) released the Crabtree effect (LOOMIS and LIPMAN 1948). Evidence for Pi involvement also comes from the demonstration that [Pi] is lowered during glycolysis (ACS and STRAUB 1954, RACKER 1956, HESS and CHANCE 1959). Raising the level of Pi in the medium reduced the Crabtree effect (BRIN and McKEE 1956, WU and RACKER 1959). Also Pi limitation in reconstituted systems can cause respiratory inhibition (GATT and RACKER 1959).

The importance of ADP limitation in respiratory repression is indicated by evidence that ADP is present in a low concentration endogenously prior to glucose addition and remains at an equally low or lower level after glucose addition (IBSEN, COE and McKEE 1958, LYNEN, 1958, HESS and CHANCE 1961). It was also shown that in isolated mitochondria ADP controls respiration much more effectively than Pi (CHANCE and HESS 1959). The transitory respiratory stimulation observed by CHANCE and HESS (1956) preceeding repression has been likened to the effect of ADP addition to ADP limited mitochondria (IBSEN 1961). All of the above work both on Pi and ADP limitation was carried out on Ehrlich ascites cells, a mouse tumor line, perhaps with an eye to deciphering the enhanced glycolytic abilities of these neoplastic cells. Subsequent to the excellent review of the Crabtree effect by IBSEN (1961) the interest of cancer researchers in this area waned.

Recently, due to increased interest in the control of mitochondriogenesis, induction and repression of respiration are again enjoying intense experimental scrutiny. Although recent investigations have focused on all aspects of repression, starting with hexose transport, most work has centred around the synthesis and assembly of mitochondrial structures.

Some controversy exists as to the mechanisms of glucose transport in yeast and as to whether or not transport is coupled to metabolism (via phosphorylation) (for a recent review of sugar transport see JENNINGS 1974). VAN STEVENINCK and ROTHSTEIN (1956) suggested that transport occurs by two systems, either by a high affinity energized system and/or by facilitated diffusion. Controversy centres around the claim that transport is coupled to phosphorylation of the sugar (VAN STEVENINCK 1968). This contention is based on evidence that glycolytically blocked cells take up a limited amount of glucose rapidly, but no free glucose can be found in the cells (VAN STEVENINCK 1969). Cells fed 2 deoxy-glucose contain both the deoxysugar and its phosphate. VAN STEVENICK (1968) reported that the sugar phosphate was a precursor for the free sugar in his experiments, thus justifying his hypothesis.

The view that phosphorylation is coupled to transport is not held by CIRILLO (1962) or KOTYK (for review see KOTYK 1973). Rather, Kotyk and co-workers believe that three monosaccaride transport systems exist in yeast. One has a broad range, transporting all monosaccharides tested. The second system is more specific for glucose-like sugars and can equalize internal and external sugar concentrations (KOTYK 1965, KOTYK 1967). And the third system operates at low glucose concentrations (0.02 - 2.0 mM) and can concentrate intracellular glucose to 85 times that in the extracellular environment (KOTYK and MICHALJANICOVA, 1968).

Despite the controversy over the transport mechanism(s) the primary metabolic consequence of sugar transport in yeast is the utilization of ATP to produce sugar phosphate (see below).

Interest in the Crabtree effect in yeast has not been as extensive as in tumors. The area of greatest interest in yeast cells has been the Pasteur effect. Over the past forty years interest in the phenomenon has

sporadically risen and waned several times and the status of the field over this period may be chronologically learned through the review and discussion articles that have appeared with regularity (BURK 1939; LYNEN, HARTMANN, NETTER and SCHUEGRAF, 1959; KREBS, 1972; RACKER, 1974). RACKER (1974) points out that the complete basis of the Pasteur phenomenon is not yet understood. Since ultimately the Pasteur and Crabtree phenomena must be viewed as reciprocals of each other, areas of experimental understanding in the Pasteur effect carry great significance with respect to the present discussion. Of fundamental significance are the contentions of LYNEN et al (1959) based on their work in yeast:

a. intracellular compartmentation of adenine nucleotides plays a primary role in regulating reactions generating or utilizing ATP.

b. important cytosolical allosteric enzymes (eg. hexokinase, phosphofructokinase) are also compartmentalized and physically removed from each other providing separate glycolytic controls cross-affected by the state of localized adenine nucleotide pools (see ascites system above).

Since in yeast hexokinase is not inhibited by glucose-6-phosphate and in muscle ATP does not directly inhibit phosphofructokinase (RAKER 1974), one other condition should be added:

c. local [Pi] can coordinate energy metabolism via allosteric effects:

(i) glyceraldehyde-3-phosphate oxidation

(ii) counteracting ATP inhibition of phosphofructokinase

(iii) counteracting glucose-6-phosphate inhibition of hexokinase

RACKER also lists:

d. citrate as an allosteric feedback inhibitor of phosphofructokinase.

While these control functions (a → d) are described in the context of glycolytic limitation in the presence of enhanced respiratory activity

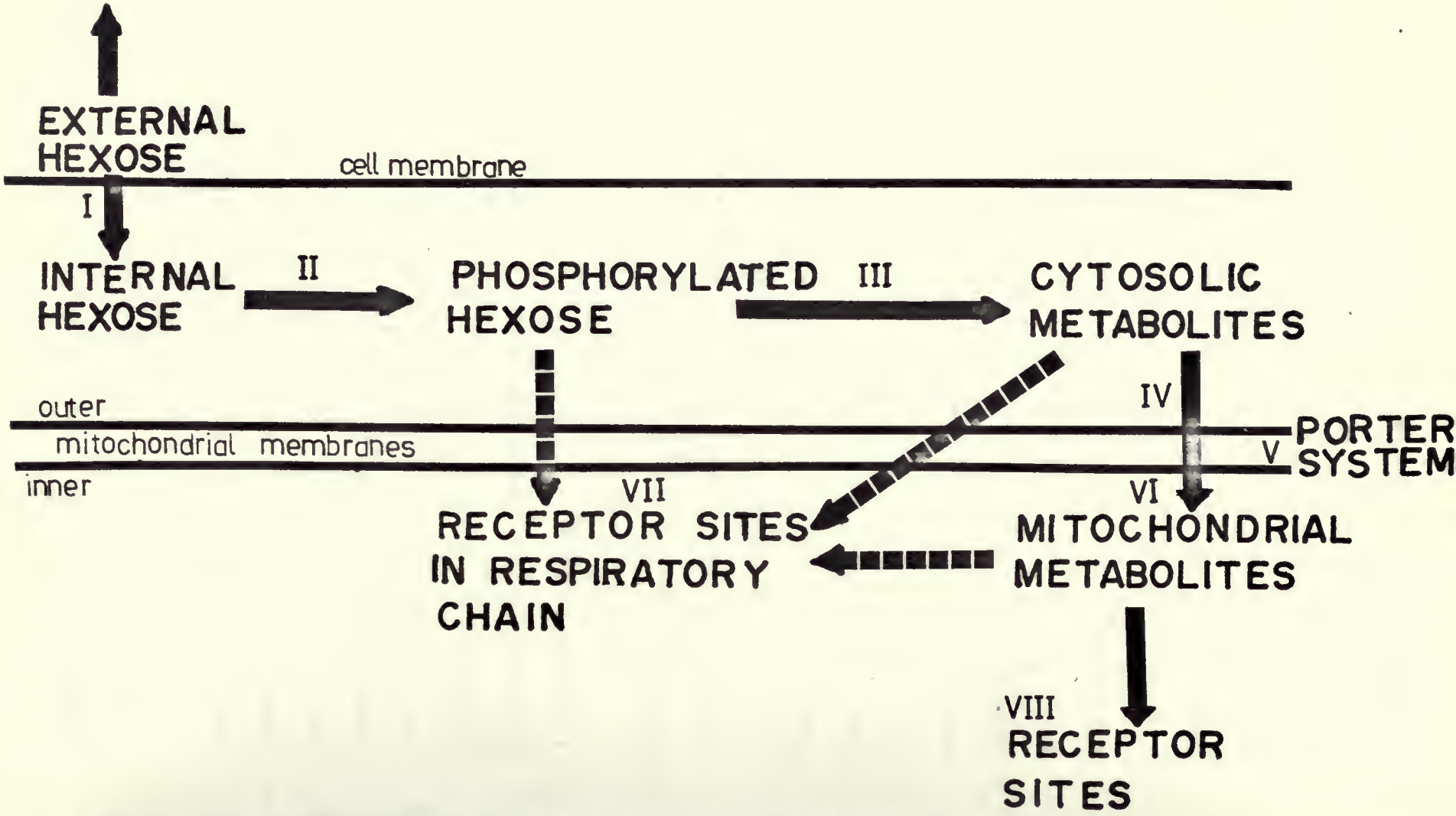
(Pasteur effect) the very bidirectionality of such controls indicates that they are probably fundamental to an explanation of the Crabtree effect.

The Crabtree effect, when elicited by the addition of small amounts of hexose to respiring cells (yeast or mammalian) is a transient phenomenon. If however, yeast are exposed to excess hexose for a prolonged time, this inhibition of respiration results in a decreased rate of mitochondriogenesis. This phenomenon, called glucose repression was first reported by TUSTANOFF and BARTLEY (1964).

B. Glucose Repression in Yeast

Yeast grown at high concentrations (5%) of glucose not only show inhibition of mitochondrial function (respiration) but lack differentiated mitochondrial structures (POLAKIS, BARTLEY and MEEK, 1964). This long lasting (relative to the Crabtree effect) glucose repression is finally reversed when glucose in the medium becomes depleted (due to fermentation) and ethanol or acetate utilization begins. At this point mitochondria appear and levels of tricarboxcylic acid cycle (TCA) and respiratory enzymes rise (POLAKIS et al 1964). The rate of induction of respiratory enzymes was shown to be inversely proportional to the glucose concentration of the medium while other fermentative carbon sources (eg. galactose) were not as repressive (POLAKIS, BARTLEY and MEEK, 1965). Similar results were reported by JAYARAMAN, COTMAN, MAHLER and SHARP (1966), and by UTTER, DUELL and BERNOFSKY (1968). During repression, [ATP] and [AMP] were high with [ADP] very low (POLAKIS and BARTLEY 1966 cf. ascites cells, IBSEN 1961). BALL and TUSTANOFF (1970) showed that high levels of reduced nicotinamide adenine nucleotide (NADH) were also characteristic of repressed cells which are unable to transfer reducing equivalents to the respiratory chain. It was suggested by these authors and by POLAKIS and BARTLEY (1965) that in

Fig. 1 POSSIBLE SITES OF MUTATIONS CONFERRING GLUCOSAMINE RESISTANCE*.



* see accompanying text for detailed explanation and discussion

addition to [ATP]/[ADP] [Pi], pyruvate and acetaldehyde may play a role in controlling the synthesis of mitochondria.

Glucose repression reverses when glucose is depleted from the medium (see above). CHAPMAN and BARTLEY (1968) reported that respiring yeast placed in 10% glucose underwent a severe respiratory inhibition and loss of mitochondrial structures which did not reverse since the substrate (glucose) was not depleted. A very large and extensive body of literature exists documenting in detail the modification of yeast mitochondria by glucose repression. The reader is referred to recent articles by LINNANE and HASLAM (1970), and PERLMAN and MAHLER (1974) for further details.

A chain of events leading to respiratory repression may occur as the following sequence. With the addition of excess hexose (glucose) to the the growth medium, transport of glucose into the cell is stimulated (Fig. 1,I). The glucose is phosphorylated via hexokinase (Fig. 1,II) leading to cytosolic ATP depletion and a concurrent rise in ADP. This lowered cytosolical ATP/ADP balance stimulates exchange of mitochondrial ATP (Fig. 1, VI) for cytosolic ADP (Fig. 1, IV) via the mitochondrial adenine nucleotide shuttle (Fig. 1, V). The influx of ADP into the mitochondrion may contribute to a transient increase in respiratory activity due to increased availability of substrates (ADP, Pi) for the ATPase (Fig. 1,VII). This may lead to mitochondrial Pi depletion.

As glucose increases the flux of metabolites through pyruvate kinase and glycerol-3-phosphate dehydrogenase (Fig. 1,III) a depletion of cytosolic Pi occurs exaggerating mitochondrial Pi depletion. This would cause severe repression of respiratory activity and perhaps in cases of extended repression lead to a termination of the synthesis of mitochondrial structures via as yet undefined mediator(s) (Fig. 1,VIII)

perhaps related to mitochondrial protein synthesis.

The mutants discussed below are resistant to poisoning by the glucose analogue D(+) glucosamine. The ability of glucose analogues to induce respiratory inhibition has been extensively recorded with D(+) glucosamine and 2 deoxy D-glucose having proven particularly useful due to their limited utilization via glycolysis (IBSEN, 1961; LETANSKY, 1968; BIELY, KRATKY and BAUR, 1974; ERRINGTON and BALL, 1974). The non-transitory nature of respiratory inhibition in the presence of glucosamine may be due to non-recycling of Pi from glucosamine phosphates which accumulate or enter other biosynthetic pathways (LETANSKY 1968), such as chitin synthesis in yeast (CABIB and KELLER, 1971). Little or no glucosamine is metabolized to pyruvate since glucosamine-6-phosphate cannot act as a substrate for glucose phosphate isomerase (BESSELL and THOMAS, 1973). No recovery from glucosamine induced respiratory repression occurs when ATP is depleted although in yeast recovery can occur with sub-lethal doses of glucosamine (ERRINGTON and BALL 1974). These authors attribute the recovery to glucosamine exhaustion.

In summary, one may conclude that resistance to glucosamine poisoning, via mutational neutralisation of the Crabtree Effect, could be due either to (i) prevention of glucosamine utilisation (Fig. 1,I,II) or (ii) failure of yeast to respond to the metabolic signals generated by (i) as described above (also see Fig. 1, III → VIII).

Clarifying Note

1. The Crabtree Effect - is an inhibition of respiration by the the addition of exogenous glucose.

2. Glucose Repression - is the repression of synthesis of the subunits (proteins, lipids) of which mitochondrial structures consist, during growth on medium containing high (5%-10%) glucose concentrations.

3. Glucosamine and the Crabtree Effect - Glucosamine induced inhibition of respiration mimics the Crabtree effect by inhibiting respiration in medium containing respiratory carbon sources (eg. glycerol). Since glucosamine is non-metabolizable growth is also inhibited.

4. Glucosamine and Glucose Repression - Since glucosamine is not metabolizable, the relationship between glucose repression and glucosamine induced respiratory inhibition is not clear. Growth on glucosamine containing medium being inhibited, the parallel situation to growth on high glucose concentration does not occur.

C. The Life Cycle and Genetics of Yeast

With much of the physiological and biochemical data pertaining to the Crabtree effect comming from mammalian systems, one may question the choice of Saccharomyces cerevisiae as the vehicle for this study. The absence of a readily accessible sexual cycle in mammalian cells essentially precludes detailed genetic analysis of loci involved in the control of this phenomenon. The eukaryotic cell system of choice has to have a simple sexual cycle, short generation time, require a minimum of special culture media or techniques and most importantly be genetically and physiologically well defined in the literature. In addition to fulfilling these criteria S. cerevisiae exhibits an isomorphic alternation of generations allowing determination of the haploid, diploid heterozygous and diploid homozygous states of a mutation with great ease.

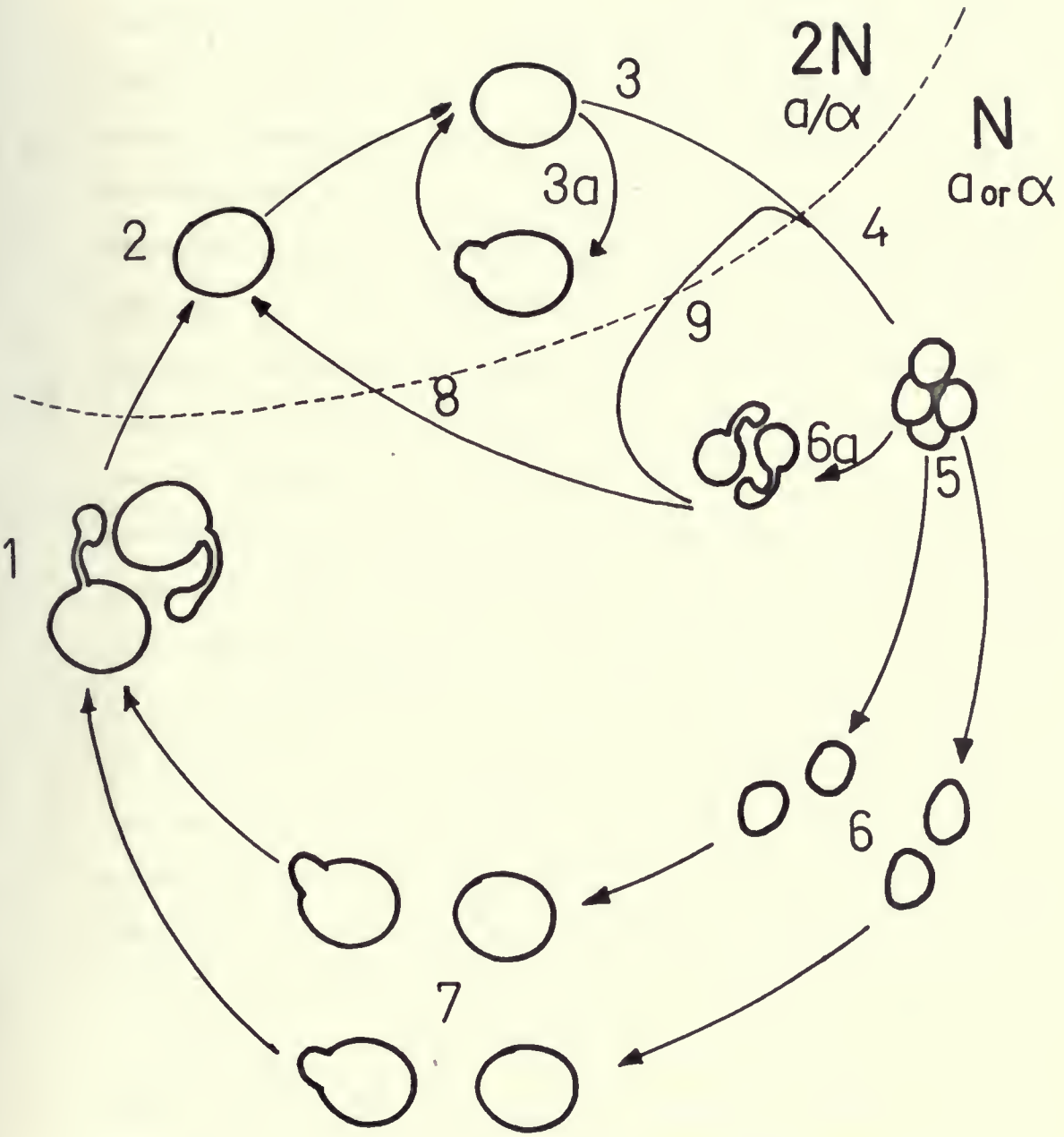
The life cycle of Saccharomyces (Fig. 2) was first described by HANSEN and WINGE (1935) followed shortly by the first description of Mendelian segregation in yeast by WINGE and LAUSTEN (1939).

In the ensuing 35 years interest in yeast genetics has increased greatly. This was partially due to the many advantages for genetic study, including rapid growth, clonability, and adaptability to techniques of replica plating and micromanipulation. To summarize the progress, both procedural and informational in yeast genetics over the past three and one half decades would by far exceed the scope and intent of the present work. Be it suffice to say that to date 143 primary functional genes have been described forming 17 linkage groups (for a recent linkage map see Appendix Ia). Several excellent reviews have appeared recently summarizing knowledge and methods in the field and the serious student in this area is referred to these (MORTIMER and HAWTHORNE 1966, HAWTHORNE and MORTIMER 1968, MORTIMER and HAWTHORNE 1969, HARTWELL 1970, FOGEL and MORTIMER 1971).

Figure 2. The Life Cycle of Saccharomyces cerevisiae*

(1) Mating type a and mating type α haploid cells mate forming (2) the diploid zygote which may either (3) enter meiosis or (3a) undergo vegetative proliferation (mitosis). During meiosis (4) an ascus is formed containing four haploid spores, two of each mating type. The ascus (5) liberates the spores which may either (6) + (7) grow as vegetative haploids or (6a) in the case of homothallic strains, individual spores produce buds of opposite mating type which may then mate with their mother cells producing zygotes which may (8) then grow vegetatively or (9) enter sporulation.

* Modified from MORTIMER and HAWTHORNE, 1969.


2N
a/α
N
a or α
1
2
3
3a
4
5
6
6a
7
8
9

D. Nuclear Mutations Affecting Mitochondrial Functions.

Mendelian mutations affecting mitochondrial function have significance to the present work and thus merit discussion here. Though CHEN, EPHRUSSI and HOTTINGUER described a Mendelian mutation preventing respiratory activity in yeast in 1950, the first comprehensive genetic study of this class of mutants was carried out 13 years later by SHERMAN (1963). He described nine nonallelic loci designated $\underline{p}_1$, $\underline{p}_2$, ...$\underline{p}_7$, $\underline{\underline{lys}}_8$. Additional p mutations have subsequently been described (REILLY and SHERMAN, 1965; HAWTHORNE and MORTIMER, 1968). SHERMAN and SLONIMSKI (1964) undertook the biochemical characterization of the original nine (SHERMAN 1963) mutants. Those designated $\underline{p}$ or $\underline{\underline{pet}}$ displayed a usual petite (see discussion of petite below in section on mitochondrial genetics) phenotype; $\underline{\underline{lys}}_6$ and $\underline{\underline{lys}}_8$ ($\underline{\underline{ly}}_6$ and $\underline{\underline{ly}}_8$) denote two genes resulting in both a lysine requirement and respiratory deficiency. SHERMAN and SLONIMSKI (1964) showed that $\underline{p}_1$, $\underline{p}_6$ and $\underline{p}_7$ lack cytochromes a, a_3 and b (cytochrome c_1 was not considered). Strains having the mutation $\underline{p}_4$ respire, have all the cytochromes but are defective utilizers of nonfementable substrates (presumbed to be oxidative phosphorylation mutants); $\underline{p}_5$ mutants lack cytochromes a and a_3. The cytochrome spectra of the $\underline{p}_2$, $\underline{p}_3$, $\underline{\underline{ly}}_6$ and $\underline{\underline{ly}}_8$ mutations were not examined since the strains were extremely unstable. Since all of the above strains had some cytochromes they are not analagous to mutant strains deficient in protoporphyrin biosynthesis (YCAS and STARR, 1953; SANDERS, MIED, BRIQUET, HERNANDEZ-RODRIGUEZ, GOTTAL and MATTOON, 1973). It was suggested by SHERMAN and SLONIMSKI (1964) that the pleiotropic nature of some of the p ($\underline{p}_3$, $\underline{p}_4$, $\underline{\underline{ly}}_6$, $\underline{\underline{ly}}_8$) mutations may be due to the alteration of some structural components of the mitochondrial membrane responsible for integration of the cytochromes into the respiratory system. About 20 "miscellaneous" mutants also described as "altered

energy metabolism" or aem have been reviewed by BECK, PARKER, BALCAVAGE and MATOON (1971).

SHERMAN (1964) has also described a class of Mendelian cytochrome mutants that unlike p mutants can grow slowly on glycerol. These mutants are designated cy and are deficient in cytochrome c activity. Genetic and biochemical characterization (SHERMAN 1964) showed that these mutations can occur at at least six distinct chromosomal loci cy_1 ..... cy_6 resulting in various levels of cytochrome c deficiencies. A continued interest in the biosynthesis and genetics of cytochrome c has given rise to much information in this area (reviewed by SHERMAN and STEWART 1971).

A significant beginning has been made in the isolation of biochemical mutants of oxidative phosphorylation (for review see KOVAC 1974). Mutants have been isolated with greatly reduced energy transfer capabilities (ie. Pi - ATP exchange) (GROOT, KOVAC and SCHATZ, 1971), inhibitor resistant ATP-ADP membrane translocator (PERKINS, HASLAM, KLYCE and LINNANE, 1973), and a mutant class has been described which not only lacks oligomycin sensitive ATPase activity but also two major F_1 subunits (EBNER and SCHATZ 1973, EBNER, MENNUCCI and SCHATZ, 1973). All of the above mutations display Mendelian inheritance, implicating a cytosolic origin for the majority of mitochondrial polypeptides (for review see SHATZ and MASON 1974).

E. Mitochondrial Genetics

It was the description of the "petite" mutation that actually "broke the ground" for the proliferation of studies into the nature of mitochondrial inheritance. EPHRUSSI and HOTTINGUER (1950) described a yeast mutant which formed only small colonies (hence petite) on medium containing fermentable

carbon sources. It was shown that though this mutation may arise spontaneously in vegetative culture (frequency about 1%), petites could be induced with 100% efficiency by the dye euflavin. Later, other agents shown to be effective were U V irradiation (PITTMAN 1959), thermal shock (SHERMAN 1959) and most effective, ethidium bromide (EB) (SLONIMSKI, PERRODIN and CROFT 1968).

The petite clones first isolated were stable in vegetative growth and when backcrossed to a wild type formed normal diploid colonies (ie. behaved like nuclear recessives). However, the petite phenotype did not segregate at meiosis and all tetrads from such crosses gave wild type spores (4:0, grands:petites) (EPHRUSSI and HOTTINGUER 1951).

The basis of the petite (rho$^-$ or ρ^-) phenotype, in addition to small colony size on fermentable media (glucose, galactose, etc.) is an inability to grow on respiratory carbon sources (eg. acetate, lactate, glycerol, ethanol) due to loss of respiratory ability. Cytoplasmic rho$^-$ strains have been shown to lack cytochromes a/a_3 b and c_1 which are essential components of the respiratory chain (EPHRUSSI and SLONIMSKI 1950). Nuclear or segregational petites (also pet or p mutants) were shown to lack cytochromes a or a_3 and b. Some nuclear petites lack cytochrome c but normal levels are found in cytoplasmic petites. (EPHRUSSI and SLONIMSKI, 1950; SHERMAN and SLONIMSKI, 1964).

A class of cytoplasmic petites displaying inheritance patterns distinct from those above were described by EPHRUSSI, MARGERIE-HOTTINGUER and ROMAN (1955). Termed suppressives, these petites showed varying vegetative and meiotic segregational patterns when mated to wild type yeast. If zygotes were sporulated immediately, tetrads yielded 4:0 rho$^-$: rho$^+$ spores indicative of a type of cytoplasmic dominance. It has been

shown that suppressive petites have detectably altered mitochondrial DNA (mtDNA), while neutral (non-suppressive) petites completely lack detectable levels of mtDNA (MEHROTA and MAHLER, 1968; NAGLEY and LINNANE, 1970; MICHAELIS DOUGLAS, TSAI and CRIDDLE, 1971).

The discovery of cytoplasmically inherited resistance to erythromycin (E^R) was reported by LINNANE, SAUNDERS, GINGOLD and LUKINS (1968) and THOMAS and WILKIE (1968). LINNANE (1968) reported a cross of erythromycin resistant to erythromycin sensitive (E^R x E^S) strains which yielded diploid clones (derived from zygotes) that were of both cell types. Subculture of E^R and E^S isolates yielded pure cell lines which when sporulated, formed tetrads containing four spores of the same type as the pure isolate (ie. 4:0, E^R:E^S for resistant isolates and 0:4, E^R:E^S for sensitive isolates). THOMAS and WILKIE (1968a) reported 3:1 and 1:3 ratios as well as 4:0 but did not specify the number of vegetative generations before sporulation was induced. Selection for E^R or E^S lines was not described prior to sporulation, suggesting that perhaps zygotes or early diploids containing a mixture of E^R and E^S cytoplasmic determinants were sporulated.

The patterns delineated in these studies of cytoplasmic inheritance of E^R became the basis for comparison of vegetative and sexual assortment for suspected cytoplasmic characteristics in yeast. THOMAS and WILKIE (1968 b) described cytoplasmic resistance to erythromycin (E), spiromycin (S) and paromycin (P) in a number of strains. They showed that drug resistance was lost by petite induction in multiple resistance strains by back-crossing induced rho^- strains to sensitive rho^+ strains. Resistance and sensitivity were found to segregate in diploid clones and recombinant drug resistant classes of diploids were also described.

LINNANE's group showed reassortment of E^R and respiratory competence in similar crosses (GINGOLD, SAUNDERS, LUKINS and LINNANE, 1969) and also described a number of additional cytoplasmic drug resistant mutants (BUNN, MITCHELL, LUKINS and LINNANE, 1970). Mutants were described with resistance to spiromycin (S), paromycin (P), chloramphenicol (C), mikamycin (M), lincomycin (L), carbomycin (Ca), oleandomycin (O), and tetracycline (T).

Slonimski's group subsequently made very major contributions toward an understanding of the phenomenology of mitochondrial recombination. COEN, DEUTSCH, NETTER, PETROCHILO and SLONIMSKI (1970) described a series of one factor crosses between E^R x E^S and C^R x C^S strains. Several strains of each mitochondrial genotype were used. Scoring for degree of transmission of drug resistance (D^R) to zygotes showed large variation depending on which strains were crossed. Strains that transmitted D^R with a high frequency (50% - 100%) to diploid vegetative segregants were said to have a high polarity in their mitochondrial genome, while those that showed low transmission frequency were designated low polarity mitochondrial genomes.

The basis of polarity was further investigated by BOLOTIN, COEN, DEUTSCH, DUJON, NETTER, PETROCHILO, and SLONIMSKI (1971). These workers postulated that polarity was due to two mitochondrial determinants $\omega+$ and $\omega-$. Preferrential transmission of mitochondrial genes was from $\omega+$ strains to $\omega-$ strains. The presence of $\omega+$ or $\omega-$ was said to be independent of cellular mating type. In homosexual crosses ($\omega+$ x $\omega+$) or ($\omega-$ x $\omega-$) parental and recombinant clones arise 1:1. The nature and existence of polarity has been disputed by RANK and BECH-HANSEN (1972) and LINNANE, HOWELL and LUKINS (1974) who have not been able to unambiguously demon-

other, functionally unrelated antibiotics (MOLLOY, HOWELL, PLUMMER, LINNANE and LUKINS, 1973; HOWELL, MOLLOY, LINNANE and LUKINS, 1974). Mutation to drug resistance occurs at two levels; alteration of mitochondrial ribosomes such as in E^R (LINNANE et al 1968 a) or changes in mitochondrial membranes to exclude the entrance of antibiotics into the mitochondrion (BUNN, MITCHELL, LUKINS, and LINNANE, 1970). Ribosomes of the former class have been shown to possess drug resistance in vitro (MOLLOY et al 1973), while those of the latter class have sensitive ribosomes in vitro (MITCHELL, BUNN, LUKINS and LINNANE, 1972).

Complete understanding does not exist as to what extent mitochondrial DNA codes for mitochondrial functions (for review see SCHATZ and MASON 1974). Genetic studies, attempting to characterize the mitochondrial genome may contribute toward an understanding of the function(s) of mtDNA. Linkage studies are slowly succeeding in establishing marker locus order toward eventual construction of a more complete mitochondrial linkage map (see Appendix II for recent linkage maps).

A recent hypothesis that the entire mitochondrial DNA is the rho^+ factor (DEUTSCH, DUJON, NETTER, PETROCHILLOR, SLONIMSKI, BOLOTIN-FUKUHARA and COEN, 1974) is based on the assumption that expression of mitochondrial genes is achieved through such a highly coordinated regulatory system that loss of any one gene "leads to inhibition of expression of the whole genome". This contention is supported by evidence that the whole mitochondrial genome in Hela cells is a single transcriptional unit (ALONI and ATTARDI 1971). A functional mechanism of this sort could explain the apparent difficulty in separating individual mitochondrial gene functions. It may also constitute an important control point in induction/repression of mitochondrial gene expression.

strate the existence of polarity in their yeast strains.

An interesting theory has been put forward to explain mitochondrial recombinational polarity with a corollary describing a possible mechanism for suppressiveness (PERLMAN and BIRKY 1974). Essentially, the mechanism described involves pairing between the polarity loci (ω+ and ω-) of recombining mtDNA molecules with a limited unidirectional degradation of the low polarity (ω-) strand. Repair of the degraded sequence is then accomplished using the ω+ strand as a template, leading to gene conversion. Suppressiveness may result from ω+ petites converting essential sequences in ω- rho+ strands to defective or incomplete sequences.

A more general theory has been put forward by CLARK-WALKER and MIKLOS (1974). This theory attributes petite inheritance and suppressivitity to the ability of the smaller rho$^-$ DNA to replicate at an enhanced rate. Petite formation as well as mitochondrial recombination may, it is suggested, result from excisions and insertions between homologous regions of the circular mt DNAs. It is propsed that these processes may exist for all small circular DNAs including bacterial plasmids. These theories are considered to be speculative at the present time.

Relating rho$^+$ to specific sequences of mitochondrial DNA would require saturation of the mitochondrial genome with genetic markers. Though a great number of drug resistant strains have been isolated and described (for review see HASLAM and LINNANE 1970) many are probably alterations of the same function(s) that map at only a few points. Twelve independently isolated oligomycin resistant (O^R) strains have been shown to belong to only two loci, O_I and O_{II} (AVNER, COEN, DUJON and SLONIMSKI, 1973). Several antibiotic resistant strains show a great deal of cross-resistance with

An alternate hypothesis is that the inner membrane assembly is so highly coordinated that any imbalance in synthetic sequence can cause the pleiotropic loss of respiratory function (c.f. nuclear mutants, EBNER and SCHATZ, 1973). Supporting evidence comes from the recent work of MICHELS, BLAMIRE, GOLDFINGER and MARMUR (1974) which suggests that some rho^- mtDNA's have massive deletions of mitochondrial tRNA loci, while others have massive duplications. Moreover, these loci are transcribed in such rho^- haploids. This evidence is not compatible with the theory (see above) which explains the rho^+ to rho^- transformation as being due to loss of any one single transcriptional locus. The following diagram (Fig. 3, modified from AINSWORTH, JANKI, TUSTANOFF and BALL 1974) summarizes the present state of knowledge with regard to the genetic control of mitochondriogenesis in yeast.

As mitochondrial DNA codes for only 5-10% of total mitochondrial protein (review by SCHATZ and MASON, 1974) it is clear that most of the proteins and lipids which constitute the mitochondrion must be "coded" on nuclear DNA (KOVAC, 1974). Thus these major mitochondrial components will be synthesized from nuclear DNA via cytosolic protein synthesis and metabolism. These cytosolic products are complemented by mitochondrial proteins which are products of the mitochondrial DNA, RNA, protein synthesizing system. As noted above (Section E) this mitochondrial infromation is essential to the biogenesis of mitochondria.

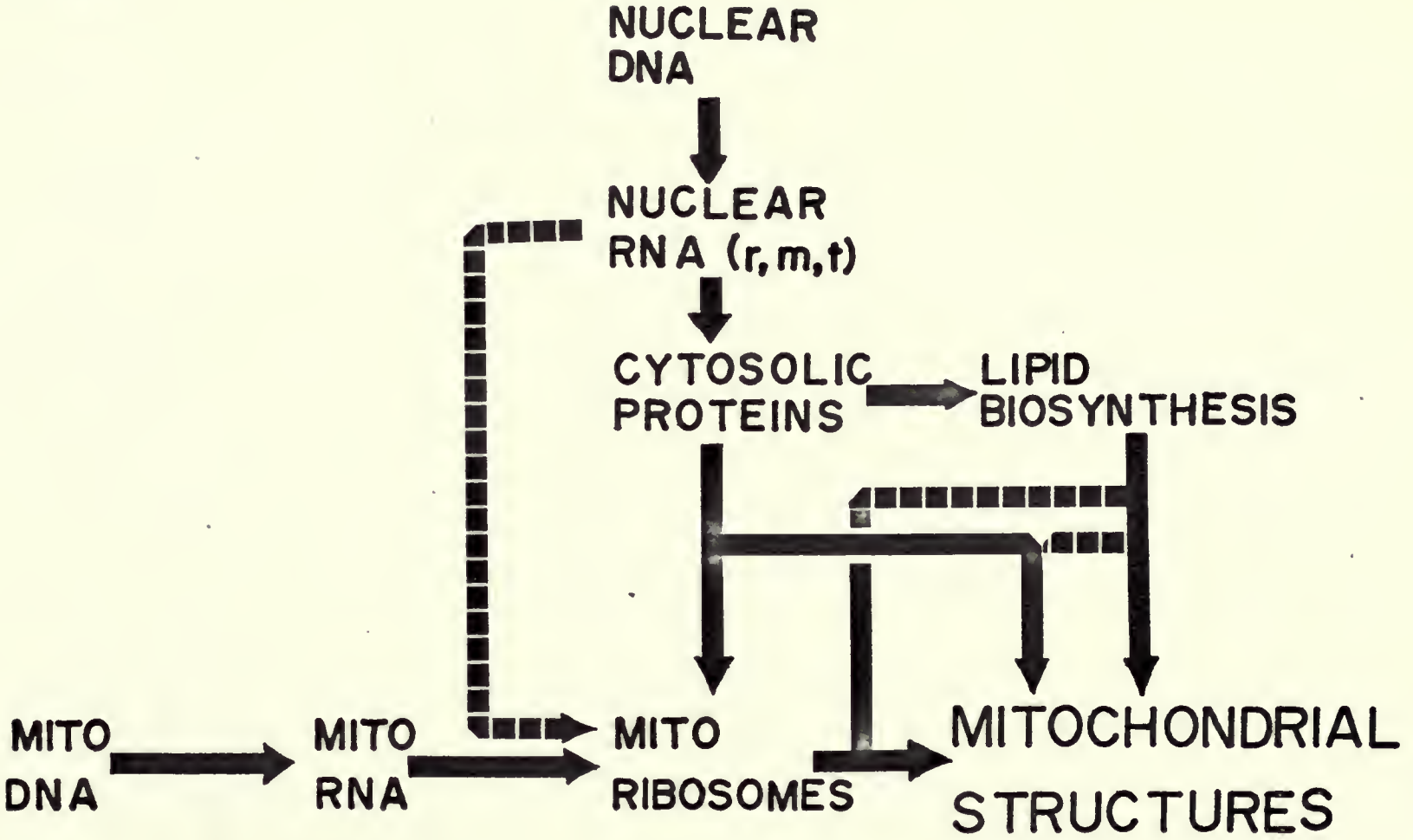
Since the effects of many nuclear and mitochondrial mutations are pleiotropic in nature (Sections D and E above), it is clear that the final assembly process wherein various lipids and proteins are integrated to form functional assemblies is complex (AINSWORTH et al, 1974). This complexity is sketched with the interconnecting lines in the bottom right

Figure 3. The Flow of Information for Mitochondrial Structures

This schematic representation of inforamtion flow is an attempt to reconcile the genetic and biochemical information available. Control points and possible feedback loops have been omitted as these are not clearly established and would complicate the diagram unnecessarily Solid lines indicate well established sequences. Broken lines represent postulated pathways or connections.
(Also see section A, B, D and E above)

Modified from AINSWORTH, JANKI, TUSTANOFF and BALL, 1974.

Fig. 3


NUCLEAR DNA
NUCLEAR RNA (r, m, t)
CYTOSOLIC PROTEINS
LIPID BIOSYNTHESIS
MITO DNA
MITO RNA
MITO RIBOSOMES
MITOCHONDRIAL STRUCTURES

hand side of Fig. 3. Although much is known about the environmental stimuli which can influence the phenomena (SHATZ and MASON, 1974), the precise metabolic events which mediate these events are still obscure.

It is hoped that the glucosamine resistant mutants described below will afford some insight into the metabolic events which mediate glucose repression and the Crabtree effect.

CHAPTER 2

MATERIALS AND METHODS

Yeast Strains

The glucosamine resistant mutant strains of haploid S. cerevisiae used in this study were produced by the ultra violet (U V) mutagenesis of two parental strains: 4B2 and 4BL. These parental strains, derived from D587-4B and D585-11C (4B2 and 4BL respectively), were a gift of Dr. Fred Sherman, Dept. Radiation Biology, Rochester University. Genotypes of the parental strains with their U V derived glucosamine resistant (GR) mutants are as follows:

4B2	α his_1	[rho+]	4BL	a lys_1	[rho+]
GR5	α his_1	[rho+]	GR112	a lys_1	[rho+]
GR6	α his_1	[rho+]	GR120	a lys_1	[rho+]
GR7	α his_1	[rho+]	GR124	a lys_1	[rho+]
GR8	α his_1	[rho+]	GR125	a lys_1	[rho+]
GR9	α his_1	[rho+]	GR127	a lys_1	[rho+]
GR10	α his_1	[rho+]	GR133	a lys_1	[rho+]
GR22	α his_1	[rho+]			
GR62	α his_1	[rho+]			

Diploids from mating (α) to (a) strains from the above are designated α strain/a strain. Thus a diploid isolate of the cross GR6 x 4BL is designated 6/4BL.

Haploid single spore GR derivatives of diploids 6/4BL, 10/4BL, and 4B2/133 used in further testing are respectively:

6L4	a lys_1	G^R	[rho+]
10P3	a lys_1	G^R	[rho+]
133F2	α his_1	G^R	[rho+]

A multiple marker strain aL1 was a gift from the Donner Research Laboratories, University of California, Berkley, California.

aL1 : a hom_2 aro_1 trp_5 leu_1 thr_1 ade_6 lys_1 his_6 ura_1 arg_{4-1} [rho+]

Additonal diploids produced and tested but not listed above are designated according to the " α strain/a strain" coding outlined above.

Media

YPD:

A complex rich medium supporting the growth of all strains: 2% glucose, 2% bacto peptone, 1% yeast extract. (All percentage concentrations are weight to volume unless otherwise stated.)

YPG:

A complex rich medium supporting growth of only respiratory competent strains: as for YPD but substitute 3% v/v glycerol for 2% glucose.

GGM:

A complex rich medium supporting growth of GR strains only: as for YPG with D(+) glucosamine hydrochloride sterilized separately and added to a final concentration of 0.05%.

SD:

A defined minimal medium supporting only prototrophic strains. It was used to select diploids formed between complementing haploid auxotrophs: 2% glucose, 0.75% Bacto yeast nitrogen base w/o amino acids.

Selective Media:

These were used to test for the amino acid requirements of isolated spore clones: as for SD with the required supplement(s) sterilized separately and added to the following concentrations:

Supplement		mg/l
histidine	(his)	20
lysine	(lys)	30
tryptophan	(trp)	20
leucine	(leu)	30
threonine	(thr)	200
adenine	(ade)	20
arginine	(arg)	20
homoserine	(hom)	30

SM:

Sporulation medium (MONTENECOURT, KUO, and LAMPEN, 1973) : 0.25% yeast extract, 0.1% glucose, 1% potassium acetate, 1.5% agar.
For solid medium 2% Difco agar was added unless otherwise stated.

Growth Conditions

Growth on solid medium was in 100 x 15 mm or 60 x 15 mm plastic petri dishes. Liquid culture was in 100 x 15 mm screw capped culture tubes containing approximately 6 ml of medium. All cultures were incubated in the dark at 30°.

Mating

Twenty-four hour YPD broth cultures of mating type a (mta) and mating type α (mt α) were mixed and inclubated at 30° for a further 4 to 6 hr. A 0.1 ml aliquot of the mating mixture was then spread onto SD solid medium. Diploid clones were evident after 48 hr at 30°.

Replica Plating

Replica plating was carried out in accordance with the method of Lederberg (LEDERBERG and LEDERBERG, 1952). When replicating a series of

plates from the same velveteen impression, plates containing rich media were replicated last.

Complementation Tests

Testing was carried out on diploid strains constructed by mating each GR strain to the parental strain (ie 4B2 or 4BL) of opposite mating type. These backcross diploid strains were transferred as point inocula to solid SD medium with not more than 27 points per plate. Master plates were replicated to GGM and control media. Plates were scored after 3 days incubation for complementation (no growth on GGM) or non-complementation (growth on GGM).

Sporulation

Diploid strains were induced to sporulate by a method modified from MONTENECOURT et al (1973). The supernatant medium was decanted from a 24 hr YPD broth culture of a diploid strain. The yeast pellet remaining was mixed into a thick suspension (with residual medium), decanted onto solid sporulation medium (50 x 15 mm plates) and spread. Sporulation was microscopically detectable after 48 hr incubation; however microdissection was only carried out after a minumum of 5 days to ensure complete maturation of spores.

Snail Gut Enzyme Preparation

Commercial snail gut preparation was diluted 1:2 with DTE buffer (1 mg/ml dithioerythritol in 0.1 M KH_2PO_4 pH, 4.5) and centrifuged 10 min at 10,000 rpm. The supernatant was then passed through a 0.22 μ Millipore filter (Millipore Corporation, Bedford, Massachusetts). Aliquots (0.2 ml) were stored at 4° in sterile screw capped glass tubes until used.

Micromanipulation and Tetrad Analysis

Ascus Digestion:

One loopful of sporulation culture was transferred to a 0.2 ml aliquot of sterile snail gut enzyme, incubated at 30° for 4 to 7 min and subsequently stored on ice.

Micromanipulation:

Tetrads were dissected according to the method described by JOHNSTON and MORTIMER (1959) using a Leitz Laborlux microscope (Ernst Leitz, Wetzlar, Germany) fitted with a X20, f = 0.40 mm objective lens (Nippon Kogaku, K.K., Tokyo). The microneedle was held in a moving stage apparatus purchased from Willian J. Hacker and Company Incorporated, West Caldwell, New Jersey.

Clones from dissected spores were transferred to YPD mater plates as point inocula to facilitate further testing.

Vegetative Segregational Analysis

After 24 hr growth in YPD broth, cells were diluted with sterile distilled H_2O and plated onto solid YPD medium at a density of 150 to 200 cells per plate. Vegetative clones arose after 48 hr incubation. Replica plating was then carried out with replicate clones scored for growth on GGM after 3 days incubation.

Ethidium Bromide Mutagenesis

Yeast were inoculated into YPD broth containing 5.0 µg/ml ethidium bromide (EB) and grown at 30° in the dark for 24 hr. Aliquots were then diluted into sterile distilled H_2O ($1:10^{-4}$) and 0.1 ml was spread onto each solid YPD plate. In 36 to 48 hr small colonies were evident on these plates which showed no growth when replicated to YPG. This method was 100% efficient in producing petites from 10P3, 6L4 and 4B2.

Cheimcals

Bacto agar, Bacto peptone, Yeast extract, Dextrose and Yeast nitrogen base w/o amino acids were purchased from Difco Laboratories, Detroit, Michigan.

Glycerol, amino acids, adenine and uracil were purchased from B.D.H. (Canada), Laboratory Chemicals Division, Toronto, and were of the highest grade available.

D(+) glucosamine hydrochloride, dithioerythritol (DTE), ethidium bromide (EB), chloramphenicol and oligomycin were purchased from Sigma Chemical Company, St. Louis, Mo.

Erythromycin gluceptate was purchased from Eli Lilly and Co. (Canada) Ltd., Toronto.

Snail gut extract was purchased from Industrie Biologique Francais, Quai du Moulin de Cage, Gennevilliers, France.

CHAPTER 3

RESULTS

Phase I - Preliminary Screening

Not every mutant strain in a study provides an equally lucrative basis for genetic analysis. Within any uncharacterized group of isolates, small or large redundancy in mutant functions may exist. The proportion of isolates carrying identical mutations must in part depend on the number of primary functions that may be altered to give the mutant phenotype. Unfortunately, some mutants may have acquired secondary mutations which affect the capacity to sporulate or maintain other faculties necessary for successful direct genetic study. Due to such considerations the initial phase of this study served a dual function. Phase I was designed to determine: (a) which mutants could be analysed and (b) the number of different loci involved. Strains to be used in subsequent phases of study were chosen on the basis of results from this preliminary analysis.

Complementation Testing

Initial attention was given to the delineation of the mutants into groups of two or more carrying the same mutation. Demonstration of glucosamine resistance (GR) in diploid isolates was to be interpreted as non-complementation and indicative of homozygosity of the GR conferring gene(s) (haploids carry the same mutation). Diploid sensitivity to glucosamine (G^S) was to be scored as complementation resulting from two haploid components carrying non-allelic mutant functions.

Construction and testing of diploids (mt α GR's and 4B2 vs mt a GR's and 4BL) for G^R or G^S did not result in definitive complementation

responses. More than half of the diploids tested gave differential responses to glucosamine within single inocula. Existence of heterogeneous haploid populations within these original isolates was excluded by subsequent extensive subcloning of all haploid GR mutants. Unambiguous complementation results could still not be obtained from diploids of these purified haploids. Almost half of the diploids tested displayed a variegated growth response when replicated to glucosamine-glycerol (GGM) medium. In such cases the conspicuous bulk of the replica inoculum displayed sensitivity but small colonies (microcolonies) within the inoculum perimeter did arise (plate 1). The number of microcolonies per inoculum varied from one to confluent growth. Scoring the later types was complicated by a need to differentiate such a response from the balanced growth evident on a homogeneous GR inoculum.

A complementation pattern was constructed including microcolony resistance as a separate category (Fig. 4). Diploids were scored as compleneting, non-complementing and microcolony non-complementing. Little information could be gleaned from the complementation pattern due to the microcolony (variegated) response which is not compatible with classical complementation theory. Vegetative segregation of phenotype is typical of non-Mendelian inheritance, therefore the GR strains were tested for vegetative segregation of glucosamine resistance.

Vegetative Segregation of G^R

Quantification of vegetative segregational patterns underlying the microcolony phenomenon was undertaken to help delineate the nature of the response. Analysis of this pattern would also help select true nuclear mutants which should not display variegated resistance patterns in isogenic cell lines. Information available on the segregational

Plate I. Growth responses of diploids to YPG and GGM medium.

a) This shows typical responses to GGM of point inocula of G^R x G^R diploids. GGM dishes were inoculated by replica plating from YPG master plates.

i) confluent growth, non-complementing, N

ii) no growth, complementing, - C

iii) micorcolony response, variegated resistance (N)

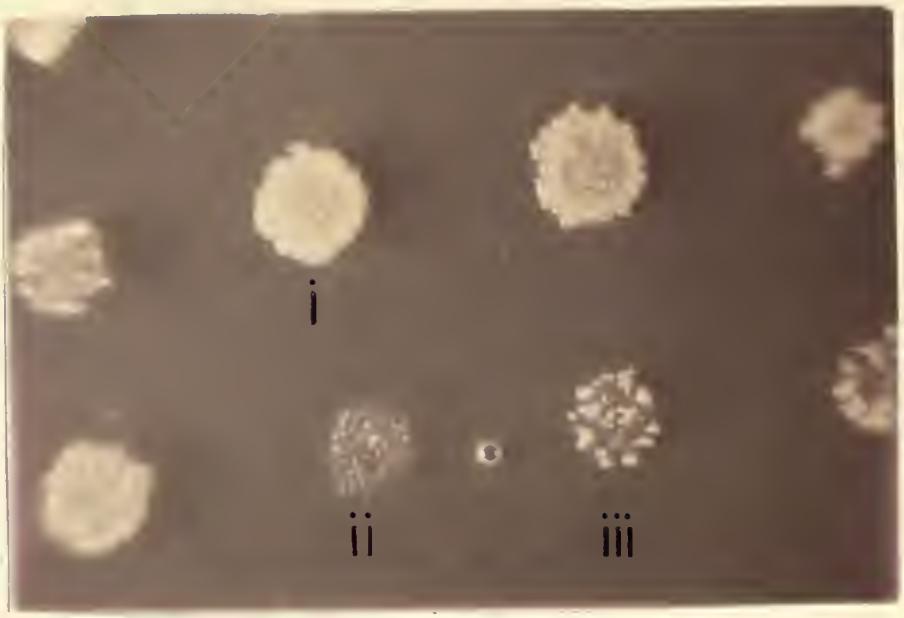
(see also Fig. 4)

b) YPG control for 6/4BL. An overnight YPD broth culture was plated onto YPD agar, giving 100-150 colonies per dish. This YPD dish was replica plated to YPG. All colonies gave the same growth response.

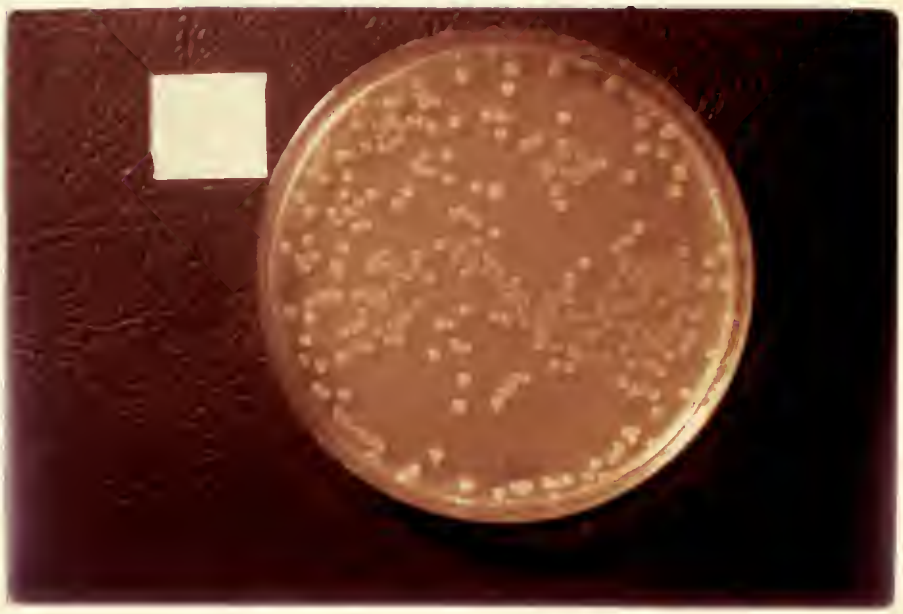
c) GGM test. This shows the variegated growth response elicited when the same colonies (plate I, b) were replica plated to GGM medium.

(see also Table I and Fig. 5)

a)



b)



c)

pp. 47

Figure 4. Complementation Pattern.

Mating type α mutants (GR5 to GR62) and the parental (4B2) were mated to the mating type a mutants (GR112 to GR133) and the parental strain (4BL). α strains appear along the top margin with a strains along the left vertical margin. Complementing (G^S) crosses are "C", non-complementing (G^R) crosses appear as "N" and variegated or microcolony resistance responses appear as (N)

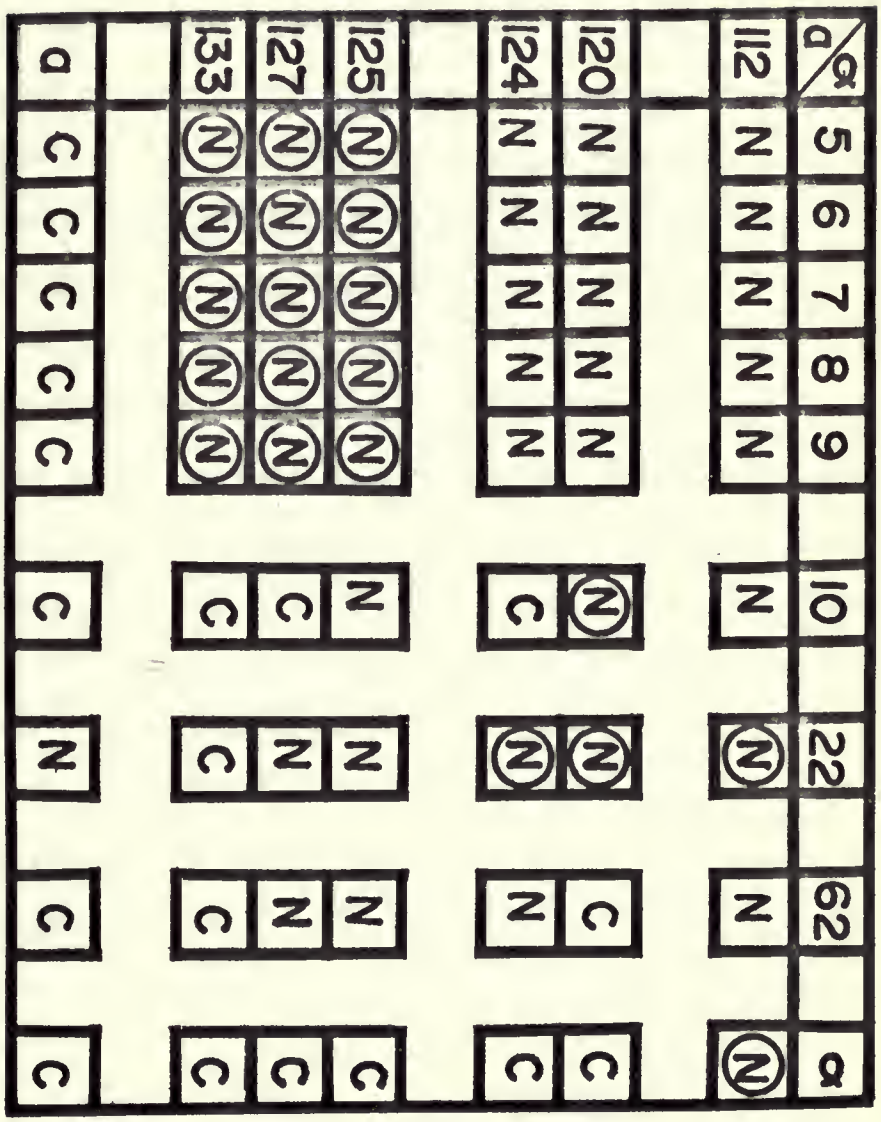


Fig. 4

patterns of mitochondrial erythromycin resistance (SAUNDERS et al, 1971) served as a model for the phenomenon of cytoplasmic inheritance.

Since GR strains were originally selected on glucosamine-containing medium,a high level of resistance was expected from the original isolates (RANK and BECH-HANSEN, 1972). It was thought that the segregational behavior of diploids (from backcross to sensitive parentals) would yield more significant information. It is the segregational behavior of this latter group that is well documented for the erythromycin resistance mentioned above. Plating experiments with 350 to 450 clones per strain scored, showed the following results: haploid isolates GR5, GR6, GR7, GR8, GR9, GR10, GR22, GR62, GR124 and GR133 each exhibited glucosamine resistance in 100% of the vegetatively derived clones scored (Table I and Table II), haploid strains GR112, GR120 and GR127 demonstrated resistance of 87%, 14% and 12% respectively (Table II). It has been established elsewhere (ELLIOT and BALL, 1973) that subcloning of resistant isolates of GR127 from such an expeirment, with subsequent replating, results in 100% resistance in the vegetatively derived clones. A similar enrichment for sensitivity is exhibited by sensitive clones derived from the same haploid line (GR127). Such selection within isogenic vegetative cell lines is characteristic of mitochondrial drug resistance mutations (SAUNDERS et al, 1971).

Attempts to measure vegetative segregation in GR125 failed due to a conditional lethality which appears in the strain. Repeated attempts at spreading turbid aliquots of culture onto YPD plates resulted in only a few heteromorphic clones which displayed a variegated response on replicating to GGM. Sub-cloning and plating of a resistant isolate of GR125 resulted in a similar pattern.

Table I. Vegetative Segregation of Resistance in 4B2 Derivatives and Respective Diploids.

GR Strain	% Resistant* Clones	Diploid	% Resistant Clones
GR5	100	5/4BL	8
GR6	100	6/4BL	16
GR7	100	7/4BL	0
GR8	100	8/4BL	40
GR9	100	9/4BL	26
GR10	100	10/4BL	100 m
GR22	100	22/4BL	100 m
GR62	100	62/4BL	0

* percent resistant clones was calculated as

(#GGM^+ clones / #YPG^+ clones) X 100, (see plate I, b and c)

m = microcolony resistance

Table II. Vegetative Segregation of Resistance in 4BL Derivatives and Respective Diploids.

GR Strain	% Resistant* Clones	Diploid	% Resistant Clones
GR112	87	4B2/112	71
GR120	14	4B2/120	1
GR124	100	4B2/124	9
GR125	---	4B2/125	29
GR127	12	4B2/127	54
GR133	100	4B2/133	96

* % resistant clones was calculated as $\frac{\#GGM^{+} \text{ clones}}{\#YPG^{+} \text{ clones}} \times 100$

(see plate I, b and c)

Zygotic clones of backcrossed GR strains all showed reduced levels of vegetative resistance (Table I and Table II). Isolates were not subjected to preferential or selective media prior to testing. This allowed unbiased segregational patterns to occur (LINNANE et al, 1968; THOMAS and WILKIE, 1968 b; BIRKY, 1973).

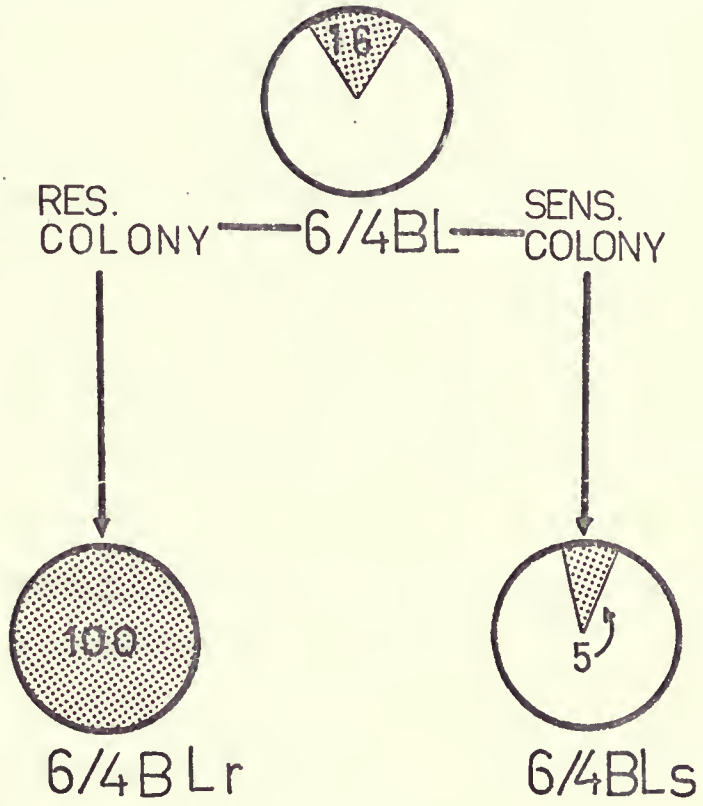
Emphasis must be given to the occurence of microcolony resistance on all diploid segregational plates. The percentage resistance of all diploids (Table I and Table II) is indicative of the percentage of vegetative clones displaying some resistance. The great majority of clones scored in these experiments as GGM^+ exhibited microcolony resistance (see Plate I). Where no apparent change took place in actual percentage of clones exhibiting resistance, comparing haploid to backcross diploid (Table I, GR10 vs 10/4BL and GR22 vs 22/4BL) the level of resistance, as judged by the number of microcolonies observed, showed a marked decrease. Haploids GR10 and GR22 gave homogeneous resistance on plating of vegetative clones. The corresponding backcross diploids (10/4BL and 22/4BL) displayed only microcolony resistance which may be interpreted as a quantitative reduction in vegetatively inheritable resistance factors.

Subcloning of resistant and sensitive colonies of 6/4BL reinforced the evidence for cytoplasmic segregation of G^R factors which may be selectively enriched or eliminated (Fig. 5) (BIRKY, 1973). Subculture of a resistant microcolony of 6/4BL termed 6/4BL r displayed a dramatic increase from the 16% resistance in 6/4BL to 100% resistance with many colonies homogeneously resistant in 6/4BLr. A corresponding decrease in resistance was achieved by subculture of a sensitive isolate termed 6/4BLs. Of all the strains tested, only GR133 showed $>95\%$

Figure 5. Vegetative Segregation of Glucosamine Resistance in a Resistant and a Sensitive Colony of 6/4BL.

The top circle represents the results of vegetative segregational analysis of 6/4BL, 16% resistance (see Table I, illustrated in Plate I, b and c). A resistant clone from a microcolony on GGM and a sensitive clone which showed no growth on GGM, were grown seperately in YPD broth overnight and plated to YPD at densities of 100 to 150 cells per dish. When colonies appeared, replica plating to YPG and GGM dishes was carried out and percentage resistance calculated. The stippled area represent % resistant clones, clear area represents % sensitive clones.

Fig. 5



resistance in both haploid and diploid vegetative cells.

Tetrad Analysis

Vegetative segregation and clonal enrichment for glucosamine resistance are not consistent with the expectations of Mendelian inheritance. These patterns do not however, provide conclusive evidence for the cytoplasmic nature of the glucosamine resistance conferring mutation. Nuclear pleiotropic mutations have been described which may induce an unstable state in certain cytoplasmic organelles (for review see BECK, PARKER, BALCAVAGE and MATTOON, 1971). Mutations of this class may be detected by analysis of the meiotic products of the backcross diploids. Tetrads produced in such a cross should yield two G^R and two G^S spores, ie. segregation of resistance should be 2:2.

If glucosamine resistance is cytoplasmically determined, tetrads would not segregate in a particular ratio but show a spectrum of tetrad classes 4:0, 3:1, 2:2, 1:3 or 0:4 since distribution of cytoplasmic determinants is not necessarily selective for G^R (LINNANE et al, 1968; THOMAS and WILKIE, 1968b). Tetrads of cytoplasmic G^R lines should show a variegated response since no selection was carried out for resistant diploids.

The data from tetrad analysis of backcross diploids is summarized in Table III. Diploids 7/4BL and 62/4BL were asporogenous, and spores dissected from tetrads of 4B2/120, 4B2/124 and 4B2/125 were non-viable and these crosses are omitted from the data. Of the remaining diploid strains only 4B2/112 exhibited below 50% spore viability.

Table III. Tetrad Analysis.

Numbers of Tetrads Scored in Each Tetrad Class (resistant:sensitive).

Strain	Tetrad Classes Scored:* 4:0	3:1	2:2	1:3	0:4	% tetrad germination
5/4BL	1	5	3	3	0	75
6/4BL	0	0	1	10	1	75
8/4BL	1	3	3	2	1	70
9/4BL	3	2	2	2	0	60
10/BL	0	1	1	7	1	55
4B2/112	1	1	2	0	0	24
4B2/127	0	1	7	1	0	60
4B2/133	0	0	7	0	0	50

* All tetrads showed 2:2 segregation for the nuclear markers his-1 and lys-1.

Veracity of tetrads was monitored through segregation of the nuclear markers $\underline{his}_1$ and $\underline{lys}_1$. All tetrads reported showed 2:2 segregation of these markers.

An unequivocally Mendelian pattern of segregation for GR was shown by 4B2/133 (Table III). Resistant segregants showed homogeneous colony resistance which could be scored 24 hr after replica plating on GGM. In contrast, all the other strains required the usual 3 days on GGM before adequate growth was attained for scoring. Resistance in segregants of these strains was of the microcolony type and did not show Mendelian segregational patterns (Table III).

Stability of Cytoplasmic G^R

A decline of resistance over storage periods was suspected when older cultures consistently showed low resistance. Cultures of GR6 and GR7 (YPD slopes) which had been utilized for vegetative segregational analysis were retested after 4 months storage at 4°. The percentage of vegetative segregants capable of producing some level of resistance had been markedly reduced (Fig. 6).

Vegetative Segregation in a 4:0, r:s Ascus

Single spore isolates from a 4:0, resistant:sensitive ascus of 8/4BL were tested for vegetative segregation of resistance. The spore lines designated 8J1, 8J2, 8J3 and 8J4 showed high levels of microcolony resistance (Fig. 7). Only 8J4 yielded sensitive isolates.

Figure 6. Stability of Glucosamine Resistance During Storage

Sub-cultures of GR6 and GR7 on YPD slopes, each displaying 100% glucosamine resistance of vegetative segregants (top circles) were stored for 4 months at 4°. The cultures were then retested by overnight growth in YPD broth, plated to YPD dishes at 100 - 150 cells/dish and replica plated to YPG and GGM. Percentage of resistant clones was calculated after 3 day growth on GGM. Stippled portion of circles represents fraction of resistant clones.

Fig. 6

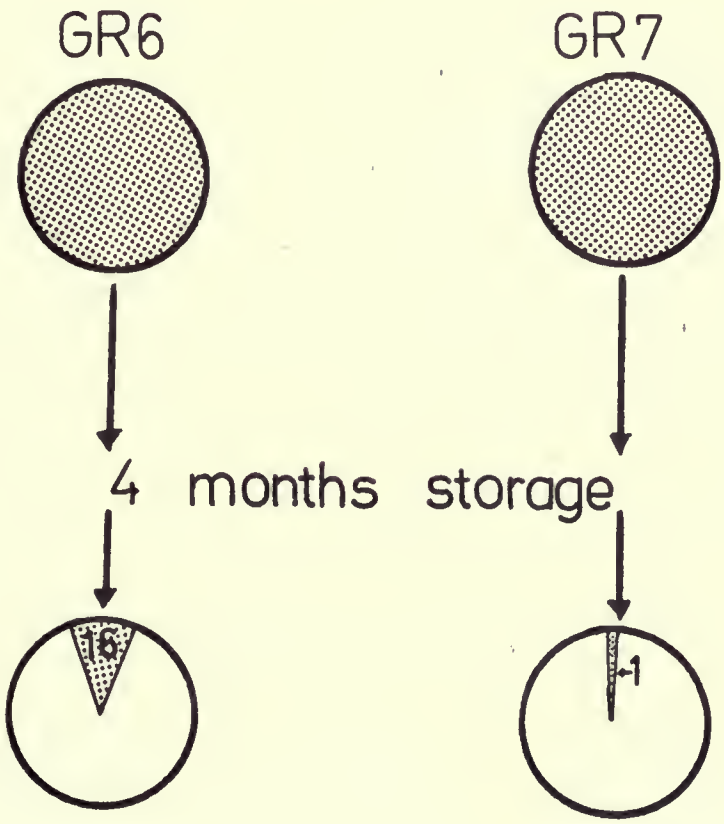
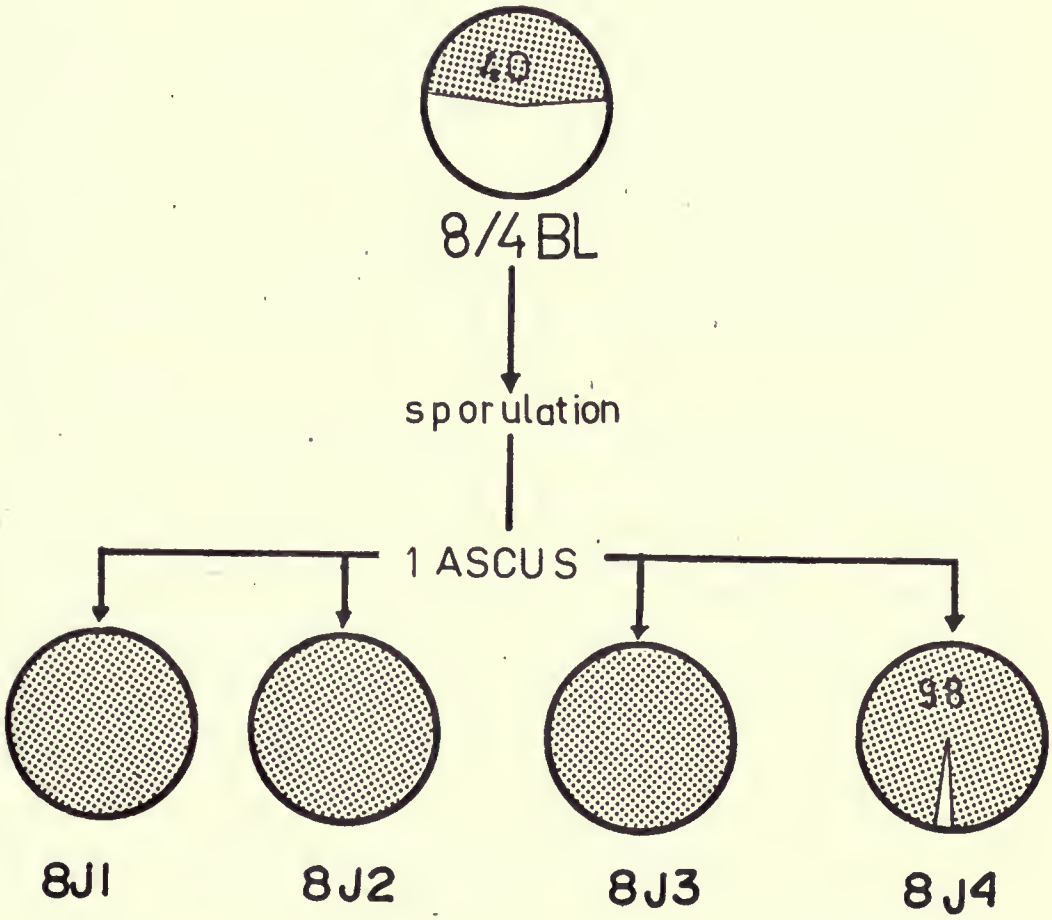


Figure 7. Vegetative Segregation of Spore Lines from a 4:0, R:S Ascus of 8/4BL.

Tetrad analysis of 8/4BL (40% resistant, top circle) yielded one 4:0, r:s ascus (Table III). Overnight YPD broth cultures of these spore colonies (8J1, 8J2, 8J3 and 8J4) were plated to YPD agar and subsequently repica plated to YPG and GGM. These plates were scored after 3 days incubation and the percentage of resistant clones calculated. Portion of clones displaying resistance are represented by the stippled fraction of circles.

Fig. 7



Phase II - Detailed Analysis

A. Linkage Study of Nuclear Mutant GR133

Only one multiple marker test strain (aL1) was available for linkage study of the locus designated gay_1 conferring glucosamine resistance in GR133. The markers carried by aL1 (see Strains in Materials and Methods) cover only a small portion of the genetic map of Saccharomyces cerevisiae, specifically parts of linkage groups VII, IX and XI (see Apendix I). Since some redundancy of map positioning was evident for the markers in aL1, only ade_6, leu_1, trp_5, lys_1, and ura_1 were scored for possible linkage to gay_1.

Mating procedure was carried out between aL1 and 133F2 (α, gay_1 his_1). Isolate 133F2 was derived from tetrad dissection of the 4B2 vs GR133 backcross. Linkage study of tetrad and spore classes are summarized in Table IV. Glucosamine resistance did not appear to be linked to any of the other markers tested or to any of the centromeres marked by these loci (Table IV).

Phase II,B - Cytoplasmic Glucosamine Resistance

Preliminary data suggest that many of the G^R mutants carry the resistance conferring gene(s) on a cytoplasmic DNA. The mutation(s) should therefore, be amenable to the same manipulations as other cytoplasmic genetic drug resistance markers (E^R, O^R etc). The following patterns should be elicitable with the proper experimental procedures.

Table IV. Linkage Study of 133R2/aL1 Tetrads.

Tetrads scored: 8

Ascospores scored: 32

Genetic markers scored*: ade_6 leu_1 trp_5 lys_1 ura_1

Locus	$X^2_{df=3}$	P	Significant at .05
ade_6	.25	>95%	no
leu_1	.25	>95%	no
trp_5	.50	>90%	no
lys_1	0	>95%	no
ura_1	.25	>95%	no

* see appendix I a & b for linkage map

1. selective enrichment for G^R cytoplasmic determinants with concomittant elimination of vegetative sensitive segregants.
2. elimination of inheritable glucosamine resistance by elimination of cytoplasmic DNA.
3. a cross to a parental with no cytoplasmic DNA should yield a diploid of homogeneous resistance which segregates only homogeneously resistant meiotic products.
4. enrichment for G^R determinants in a previously variegated backcross diploid should result in an increased, complete (ie. non-variegated) level of resistance in the diploid and all meiotic products.

Two mutants were chosen for further testing (GR6, GR10). Some physiological data existed for GR6 (ERRINGTON and BALL, 1974) providing a broader perspective for genetic study. GR10 was chosen for further study simply because 10/4BL asci were easy to dissect.

GR10

Segregational patterns of GR10 and 10/4BL in vegetative culture have already been described in previous sections (see Phase I, Vegetative Segregation; Table I and Table II). The products of a single 1:3, resistant:sensitive ascus from tetrad analysis of 10/4BL were subjected to vegetative segregational analysis. The strains were designated 10P1, 10P2, 10P3 and 10P4. A microcolony or variegated resistance response had been recorded for 10P2 in tetrad analysis. The others had been scored as glucosamine sensitive.

Vegetative segregational data (Fig. 8) indicated that with 91% of segregants containing resistant microcolonies, 10P2 showed the highest resistance level. The other strains ranged from 54% to 1.3% with 10P3 showing the latter, lowest response. A resistant microcolony of 10P3 was subcultured to GGM broth, incubated for 3 days and plated to GGM solid medium. A clone was isolated and subcultured to YPD broth for vegetative segregational analysis. All vegetative segregants of this isolate designated 10P3r displayed complete, homogeneous resistance with no microcolonies and no sensitive segregants (Fig. 8). Success of this enrichment procedure fulfills the first criterion for cytoplasmic location of this GR mutation.

Ethidium Bromide Induced Loss of GR

Growth for 24 hr in medium containing 5 μg/ml ethidium bromide (EB) should result in complete elimination of mitochondrial DNA (SAUNDERS, GINGOLD, TREMBATH, LUKINS and LINNANE, 1971).

Such treatment of 10P3r produced respiratory incompetent cells with 100% efficiency. A petite isolate (10P3ρ^-) was mated to 4B2 and the resultant diploid (4B2/10P3ρ^-) was subjected to vegetative segregational and tetrad analysis.

A dramatic quantitative drop in resistant vegetative segregants was recorded (Fig. 8) by comparison to 10/4BL. Only 2% of segregants showed microcolony resistance compared to 100% in 10/4BL. The qualitative drop in resistance was also radical. Resistant vegetative segregants of 10/4BL often contained several microcolonies on glucosamine plates, while only a single microcolony per resistant segregant was evident in 4B2/10P3ρ^-.

Figure 8. Vegetative Segregation of Glucosamine Resistance in GR10 and its Derevatives

This figure illustrates the variability and manipulability of glucosamine resistance in these strains. Each circle represents one population with the stippled portion representing the percentage of vegetative segregants that displayed resistance. Where % resistance $\leq$100 the actual value is indicated within the circle. Microcolony resistance is indicated by the appearance of M within the circle.

a. Haploid strain GR10, 100% resistant

b. Diploid strain 10/4BL, 100% resistant (microcolonies)

c. After sporulation of 10/4BL one tetrad (10P) gave rise to four sister spores which showed various degrees of resistance (10P1 $\longrightarrow$ 10P4).

d. The least resistant spore 10P3 was sub-cultured into GGM broth for 5 days and subsequently tested for glucosamine resistance. Result - 100% resistance.

e. A highly resistant clone resulting from (d) was designated as 10P3r and subjected to these treatments:

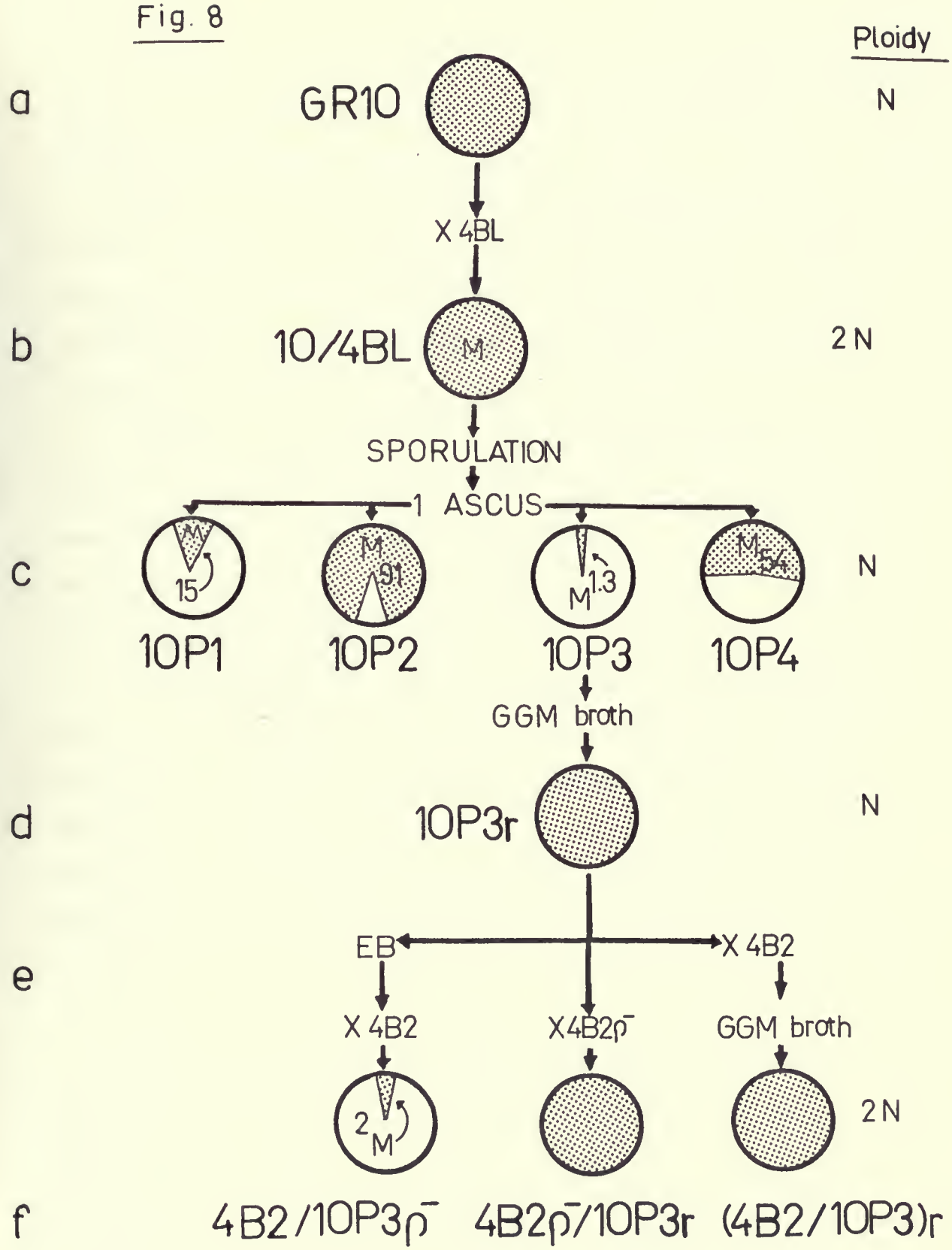
   i) elimination of mt DNA with EB followed by crossing to 4B2

   ii) a direct cross to 4B2ρ^-

   iii) crossing to 4B2 followed by passage through GGM broth.

f. The resulting diploids, 4B2/10P3ρ^-, 4B2ρ^-/10P3r and (4B2/10P3)r were tested for % resistance.

Fig. 8


Ploidy
a
GR10
N
X 4BL
b
10/4BL
M
2 N
SPORULATION
1 ASCUS
c
M
15
M
91
M
1.3
M
5+
N
10P1
10P2
10P3
10P4
GGM broth
d
10P3r
N
e
EB
X 4B2
X 4B2
X4B2ρ⁻
GGM broth
2
M
2 N
f
4B2/10P3ρ⁻
4B2ρ⁻/10P3r
(4B2/10P3)r

No resistant spores were recovered in tetrad analysis of 4B2/10P3ρ^- (Table V). All tetrads scored were 0:4, resistant:sensitive.

Passage of Resistance in Cross to Petite

Mating of higly resistant 10P3r to an EB induced petite of 4B2 (4B2ρ^-) resulted in a diploid isolate of high resistance. Sensitive clones could not be detected in vegetative platings (Fig. 6). Many segregant clones showed a homogeneous resistance response but some microcolony resistance was still evident.

Analysis of tetrads indicated a shift toward a higher ratio of resistant segregants per tetrad over 10/4BL. It was also noted that several resistant spores gave rise to lush whole inoculum resistance responses which was not evident in 10/4BL (Table V).

Enrichment of Resistance in Diploid

A diploid of 4B2 x 10P3 was isolated from a mating mixture. The diploid was inoculated into GGM broth, grown for 4 days and plated to solid GGM. A clone was isolated and designated (4B2/10P3)r. This isolate was vegetatively homogeneous in resistance (Fig. 8). Tetrads dissected all scored 4:0, resistant:sensitive with all segregants showing homogeneous, lush growth on GGM plates (Table V).

GR6

The vegetative segregational patterns of GR6 and 6/4BL have already been described in Preliminary Analysis (Table 1). Tetrad analysis of 6/4BL has also been described (Table III). Four spores of a single 1:3, resistant:sensitive ascus of 6/4BL were scored for vegetative segregation of GR. Of the isolates 6L1, 6L2, 6L3 and 6L4 it was the one which had demonstrated resistance in tetrad

Table V. Tetrad Analysis of GR10 Derivatives.*

Strain	Tetrads scored in classes (r:s) 4:0	3:1	2:2	1:3	0:4	% germination
10/4BL	0	1	1	7	1	55
4B2ρ^-/10P3r	1	6	6	2	1	80
(4B2/10P3)r	12	0	0	0	0	80
4B2/10P3ρ^-	0	0	0	0	10	75

* All tetrads showed 2:2 segregation for the nuclear makers his-1 and lys-1

analysis (6L4) which also yielded the highest value for vegetative resistance - 71% of segregant clones contained microcolonies on GGM (Fig. 9). The others demonstrated values between 40% and 0% resistant segregatns. Enriched resistance was induced in a microcolony isolate of 6L4 in the same manner described above for 10P3r. This isolate (6L4r) exhibited 100% homogeneous resistance in vegetative segregants (Fig. 9).

Ethidium Bromide Treatment of 6L4r

Treatment with EB was carried out for 6L4r as described for 10P3ρ^-, and 6L4ρ^- was isolated. Diploid isolate 4B2/6L4ρ^- resulted from the cross: 4B2 x 6L4ρ^-. Vegetative segregants of 4B2/6L4ρ^- displayed microcolony resistance in 99% of clones scored (Fig. 9). Tetrads dissected also showed relatively high resistance levels with no tetrads in the 0:4, resistant:sensitive class (Table VI). It is emphasized that all resistance was of the microcolony type and no full colony resistance was observed.

Inheritance of Resistance in Cross to a Petite

Diploid 4B2ρ^-/6L4r was isolated from a mating mixture of 4B2ρ^- and 6L4r. Homogeneous resistance was elicited from 100% of vegetative segregants (Fig. 9). Tetrad analysis suggests a shift toward greater ratios of resistance than evident in 6/4BL (Table VI). While distribution of tetrad classes did not seem to vary a great deal between 4B2/6L4ρ^- and 4B2ρ^-/6L4r, it should be stressed that qualitative growth responses were quite dissimilar. All resistant responses scored in 4B2/6L4ρ^- were of the microcolony type, often with 3 or fewer microcolonies per inoculum. Tetrads of 4B2ρ^-/6L4r yielded resistant ascospore lines which often exhibited lush whole inoculum growth in three or four segrega nt.

Figure 9. Vegetative Segregation of Glucosamine Resistance in GR6 and its Derivatives.

This figure illustrates the variability and manipulability of glucosamine resistance in these strains. Each circle represents one population with the stippled portion representing the percentage of vegetative segregants that displayed resistance. Where % resistance <100, the actual value is indicated. Microcolony resistance is indicated by the appearance of M within the circle.

a. Haploid strain GR6, 100% resistant

b. Diploid strain 6/4BL, 16% microcolony resistance.

c. After sporulation of 6/4BL one tetrad (6L) gave rise to four sister spores which showed various degrees of resistance (6L1 ⟶ 6L4).

d. The most resistant spore, 6L4 was subcultured into GGM broth for 5 days and subsequently tested for glucosamine resistance. Result - 100% resistant.

e. A highly resistant clone resulting from d. was designated 6L4r and subjected to the following treatments:

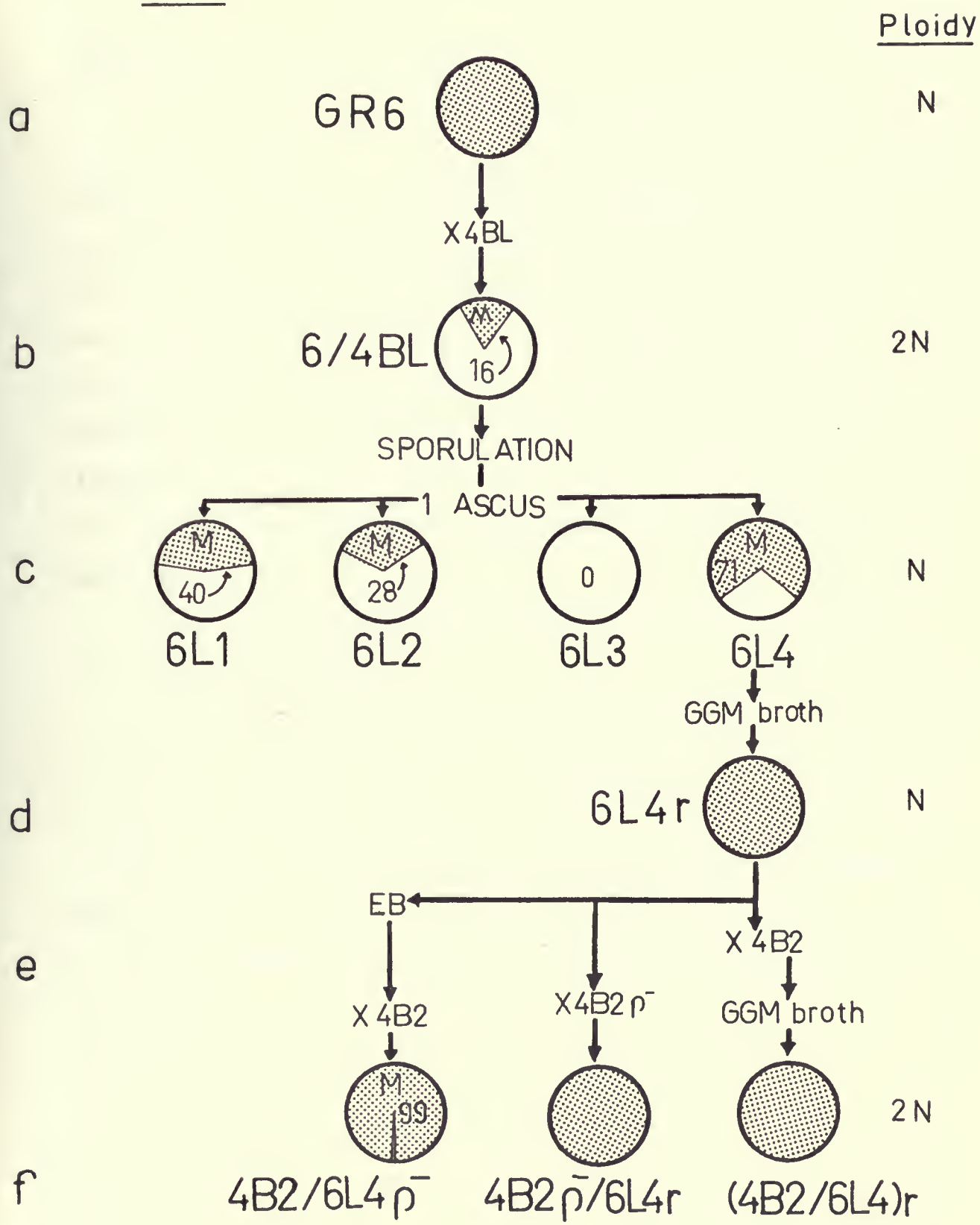
   i) elimination of mt DNA with EB followed by crossing to 4B2

   ii) a direct cross to 4B2

   iii) crossing to 4B2 followed by passage through GGM broth.

f. The resulting diploids 4B2/6L4 , 4B2 /6L4r and (4B2/6L4)r were tested for % resistance.

Fig. 9


Ploidy
a
GR6
N
X4BL
b
6/4BL
M
16
2N
SPORULATION
1 ASCUS
c
M
40
6L1
M
28
6L2
0
6L3
M
71
6L4
N
GGM broth
d
6L4r
N
EB
X4B2
e
X4B2
X4B2 ρ⁻
GGM broth
M
99
2N
f
4B2/6L4 ρ⁻
4B2 ρ⁻/6L4r
(4B2/6L4)r

Enrichment of Resistance in Diploid

Backcross of 6L4r to 4B2 resulted in isolation of a diploid which after enrichment for resistance was designated (4B2/6L4)r. All vegetative segregants were resistant with homogeneous growth on test medium (Fig. 9). Attempts to sporulate this diploid met with partial success. Ascus production was lower than 10% with complete tetrads constituting less than 1% of all cells in the sporulation culture. Asci were poorly formed and irregular in shape which hindered tetrad dissection. Of the tetrads dissected only 20% contained all viable ascospores. All tetrads scored showed 4:0, resistant:sensitive segregation. All resistance was complete and homogeneous (Table VI).

Cross Resistance

Drug resistance was determined on YPG agar with the drug added in the concentration noted.

Strains 6L4r and 10P3r were not resistant to erythromycin (5 mg/ml), oligomycin (5μg/ml), or chloramphenicol (5 mg/ml). Type cultures of mitochondrial mutants obtained from Linnane (E^R, strain L411, SAUNDERS et al, 1971), Rank ($C^R E^R$, strain 44-5a, RANK and BECH-HANSEN, 1972), and Griffiths (O_I^R, strain D22-A16, and O_{II}^R, strain D22-A13, AVNER, COEN, DUJON and SLONIMSKI, 1973) did not show cross resistance to glucosamine.

Table VI. Tetrad Analysis of GR6 Derivatives*.

Strain	Tetrads scored in classes (r:s) 4:0	3:1	2:2	1:3	0:4	% germination
6/4BL	0	0	1	10	1	75
4B2 ρ^-/6L4r	1	7	4	0	0	60
(4B2/6L4)r	3	0	0	0	0	20
4B2/6L4 ρ^-	4	2	6	1	0	65

* All tetrads showed 2:2 segregation for the nuclear markers his-1 and lys-1

CHAPTER 4

DISCUSSION

Glucosamine Resistance in the Context of the Crabtree Effect

The mechanisms leading to respiratory repression are far from clear, as discussed in the previous treatment of catabolite repression and the Crabtree effect (see Review of the Literature). Some speculation as to the relation of glucosamine induced respiratory repression to the Crabtree effect is however in order.

Due to the limited penetration of glucosamine into the cellular metabolism (see Chapter 1, section A), in all probability only three major functions exist which could alter cellular metabolism to confer glucosamine resistance:

1. a mutation affecting the cellular transporter causing limitation or loss of ability to transport external glucosamine into the cell (Fig. 1, function I)
2. a mutation affecting the ability of hexokinase to phosphorylate glucosamine into glucosamine-6-phosphate (Fig. 1, function II)
3. a mutation affecting some as yet unknown mitochondrial function(s) which mediates the interaction between hexose phosphorylation and respiratory inhibition. (Fig. 1, functions III$\longrightarrow$VIII)

Two of the above mutational classes would probably exhibit Mendelian inheritance (patterns 1 and 2) since they involve modifications in cytosolic and not mitochondrial components. These mutations may involve a modified uptake or phosphorylation response to glucosamine

specifically and therefore not be candidates for generalized resistance to the Crabtree effect. Since at the concentrations involved in these experiments, the bulk of glucosamine may enter the cell through simple diffusion and not involve a carrier (RENNER et al, 1972) it is probably unlikely that a mutation involving the membrane bound glucose carrier would affect the ability of glucosamine to diffuse across the membrane. On the other hand, a modification of hexokinase, limiting or preventing phosphorylation of glucosamine, and therefore glucosamine induced ATP and/or Pi depletion, could constitute an effective block to the chain of intracellular events leading to respiratory repression. Limitation of the phosphorylation step may also cause a backup of intracellular glucosamine, reducing the diffusion gradient and accounting for the reduced uptake observed in some mutants (ERINGTON and BALL, 1974).

Of greater significance from a functional point of view would be mutation(s) affecting the phosphate economy of the mitochondrion. The events involved in this level of mutation would directly affect oxidative phosphorylation, thus not being peculiar to glucosamine induced respiratory repression. Such mutation(s) would confer a modified Crabtree response upon cells regardless of the hexose used to elicit the effect. In this case, resistance to glucosamine may imply resistance to catabolite repression. Mutations of this nature could affect at least two functions.

3 (a). mitochondrial adenine nucleotide translocator.

3 (b). oligomycin - sensitive ATPase.

It has been demonstrated that both nuclear and mitochondrial gene functions are required for normal activity of translocator and ATPase (KOVAC and WEISSOVA, 1968; SCHATZ, 1968; AVNER and GRIFFITHS, 1973; HASLAM, PERKINS and LINNANE, 1973) for review see SCHATZ and MASON (1974) and therefore a number of nuclear and mitochondrial mutations could conceivably alter the activities of either function.

One plausible mechanism for resistance is a change in the substrate affinity of the adenine nucleotide translocator. A significant decrease in the rate of ADP translocation into the mitochondrion (and ATP out) would limit the rate at which ATP and therefore Pi depletion by hexokinase in the cytosol could occur. Modification of the mitochondrial inner membrane and/or the carrier itself could also be involved.

A modified mitochondrial ATPase (oligomycin sensitive ATPase or F_1 ATPase) could also limit phosphate depletion by making ATP production limiting. An impaired rate of ADP $\longrightarrow$ ATP would reduce Pi consumption and perhaps circumvent depletion. Mutations affecting ATPase structure could show nuclear inheritance (SCHATZ and MASON, 1974) but those involving membrane association of the ATPase-membrane complex could be the result of modificiation(s) of mtDNA (TZAGOLOFF and MEGHER, 1972).

Preliminary Analysis of Mutants

It was assumed that the majority of mutated primary functions conferring glucosamine resistance would exhibit Mendelian inheritance. The preceding discussion has indicated that besides glucose transport and hexokinase activity, the mitochondrial ATP-ADP translocator and ATPase all come under nuclear control, indeed six of ten peptides

constituting the mitochondrial ATPase are synthesized completely on cytoplasmic ribosomes with mitochondrial ribosomal products only required for membrane binding (TZAGLOFF and MEGHER, 1972; SCHATZ and MASON, 1974). Therefore in the name of expediency complementation testing was attempted between mtα and mt a mutants to circumvent the time consuming necessity of producing mt a and mtα derivatives of individual mutants. Interpretation of the resultant complementation pattern (Fig. 4) in terms of Mendelian inheritance was impossible. Neither was the pattern wholly similar to that of established mitochondrial genetic markers (E^R, O^R). The failure of all backcrosses to parental sensitives except 4B2/112 to show any resistance whatsoever in replica plating was not similar to the mixed zygote clones described for backcrosses of E^R or O^R cytoplasmic mutants (LINNANE, SAUNDERS, GINGOLD and LUKINS, 1968; AVNER and GRIFFITHS, 1970). Zygotes isolated after mating (E^R vs E^S, or O^R vs O^S) in these studies tended to purify during the first buddings to yeild a mixture of pure drug resistant or pure drug sensitive cell lines (LUKINS, TATE, SAUNDERS and LINNANE, 1973).

Complementation testing of G^R strains was first carried out on zygote clones, and then on sub-clones from streaking of the zygote lines. Neither procedure produced appreciably different results and both sets of results were used for the complementation pattern (Fig. 4). The pattern did not rule out a nuclear mode for inheritance of G^R in most of the mutants despite the microcolony resistance response shown by a large group of crosses.

The most difficult aspect of the complementation pattern to reconcile with Mendelian inheritance of G^R was the lack of a consistent pattern in any of the groups. For instance, on the basis of complementation data it may be argued that GR5, GR6, GR8, GR9, GR10, GR120 and GR124 belong to the same Mendelian complementation group. Microcolony resistance may be the result of a cross to a complementing but cytoplasmically resistant mutant. Such a rationale should still yield consistant complementation behavior for all members of the group. On this basis the resistance responses of the diploids between GR120 or GR124 and GR7 or GR62 are anomolous (Fig. 4).

The vegetative segregational and tetrad analyses carried out on all of the mutants helped to establish some of the basic inheritance patterns for the cytoplasmic G^R factor (Table I, Table II). Resistance in all mutants save GR133 seemed to be cytoplasmically inherited. The original microcolony response observed in complementation testing subsequently showed itself to be characteristic of the cytoplasmically mixed populations of diploids formed by a mating between resistant and non-resistant haploids (Table II). Again, this was atypical of the similar backcrosses of E^R vs E^S and O^R vs O^S mentioned above. In the case of cytoplasmic G^R, cytomixis seems to result in a dilution effect of the relative numbers of resistant to sensitive cytoplasmic factors. The lower level of cytoplasmic resistance does not seem sufficient to confer resistance on cells with such a cytoplasmic complement. There appears to be no intracellular selection for or against resistant cytoplasmic factors as indicated by a lack of significant variation between the microcolony resistance responses of zygote clones and subsequent vegetative isolates several vegetative generations removed. Whether the loss of cytoplasmic

G^R during cold storage (Fig. 6) is the result of intracellular selection, long term instability in resting cells, or a low temperature lability is a matter for speculation at this stage.

The microcolony response can be explained by the following rationale. It has been suggested (JAMES and SPENCER, 1958) that yeast have no mechanism for the orderly distribution of cytoplasmic determinants between buds. On this basis, it may be resonable to interpret the ontogeny of the microcolony response as the following sequence. A budding yeast strain with a mixed population of G^R and G^S determinants usually produces daughter cells with a similarly mixed complement of determinants. The G^R level, or ratio of G^R to G^S in this strain is not adequate to confer glucosamine resistance upon it. However, due to occasional unequal distribution of cytoplasm during budding some daughter cells arise with the greater G^R levels necessary to resist glucosamine induced respiratory repression. In an exponential, glucose grown population such cells appear to have no selective advantage and do not serve to increase the relative resistance levels in the population. When plated to GGM the bulk of such a cell population is growth inhibited with eventual cell death following (only microcolonies survive in a subsequent replica plating of a 3 day GGM plate to YPG). Resistant vegetative segregants (those receiving higher levels of G^R in budding) form the basis of the microcolony response by proliferating in response to the selection pressure of GGM medium to form the small visible clones here termed microcolonies. These microcolony cell lines would carry higher levels of vegetative resistance due to the greater levels of G^R inherited from the resistant founding cells. Such a pattern is indeed displayed by microcolony isolates (eg. 6/4BL and 6/4BLr in Fig. 5).

While the above hypothesis may be somewhat at variance with the patterns of intracellular selection in mitochondrial E^R mutants described by Birky (1973), it does explain microcolony resistance where E^R type mitochondrial selection would not. Birky described the development of pure E^R or E^S cell lines on the basis of intracellular selection, even in non-selective glucose medium (Thomas 1969). As already described, such selection is not evident for cytoplasmic G^R factors. Cytoplasmic GR mutants maintained a heterogeneous cytoplasmic population of G^R and G^S determinants unless placed under selection pressure for G^R (enrichment in GGM broth).

The possibility of spontaneous mutation playing a significant role in microcolony resistance has not been seriously considered for two reasons. The incidence of spontaneous mutants in parental strains is extremely low (4B2, 4BL) with none scored for spread plates containing 7,300 colonies after 3 days. Secondly, microcolonies give rise to sensitive clones with resistant microcolonies (6/4BL r, Fig. 3) indicating that the microcolony resistance response is an inherited phenomenon and not due to mutational divergence within cell lines (c.f. segregation test as applied by BIRKY, 1973). Additional evidence is supplied by the time scale: microcolony resistance is manifested between 48 and 72 hrs whereas comparable numbers of spontaneous mutants do not arise in the parental strains until 6-7 days at 30°C, if at all (c.f. BIRKY, 1973).

Inheritance and Maintenance of Cytoplasmic G^R

The requirement for some critical level or ratio of G^R determinants in a cell before it manifests glucosamine resistance is implied by the

demonstration of G^R segregants from a sensitive strain (see 10P3, Fig. 8). Cytoplasmic G^R determinants must exist in such strains (spontaneous mutants are rarely observed in the parental strains) yet are not expressed due to a low multiplicity within such cells. This is the major reason why replica plate testing of point inocula has not proved an adequate method for assessing the existence of cytoplasmic G^R factors.

Similarily it is not necessary for the cytoplasmic determinants to be 100% G^R before a glucosamine resistant phenotype can be expressed. Resistant microcolonies when subcultured to YPD broth and replated show elevated but still rather incomplete resistance responses (6/4BL r, in Fig. 5). A rather simple minded but demonstrative calculation may be used to clarify this point for 6/4BL r (see A →D next page).

Since no selection pressure was brought to bear on the microcolony isolate (6/4BLr) prior to replating, the value in (D) may be considered the upper range for occurance of cells with threshold levels of cytoplasmic G^R in a microcolony. The value range in (C) may in all probability be orders of magnitude low, thus skewing the range of values in (D) to higher than actual occurence. This calculation thus serves as rather a dramatic illustration of the heterogeneous composition of even an apparently resistant cell line (microcolony resistant).

Acceptance of such a composition for a resistant microcolony seems to consitute a paradox ie. a resistant microcolony is composed almost entirely of sensitive cells. Several factors may be involved in producing such a situation. Depletion of localized glucosamine, especially on solid medium may play an important role in the maintenance of a heterogeneous population of cells in a resistant microcolony. Both

vegetative segregants with microcolonies	100%	(A)
usual number of microcolonies per clone	3 to 5	(B)*
number of cells transferred from velvet to GGM plate per 2-4 mm clone	10^3 to 10^6	(C)+
occurance of cells with threshold resistance in microcolony isolate		(D)

$$(D) = 5/10^{3} \longrightarrow 3/10^{6} = \underline{2.0 \times 10^{-4}} \longrightarrow \underline{3.3 \times 10^{-7}}$$

* 85% of clones fell into this range, 12% showed 1 or 2 microcolonies, and 3% showed from 6 to confluent growth on GGM.

+ 10^3 to 10^6 cells is an assumed range designed to represent what may be the lower limits of transfer to allow an upper threshold value for (D).

resistant and wild type (4B2) yeast cells take up glucosamine (ERRINGTON and BALL, 1974). Within the perimeter of an inoculum on solid medium such uptake may result in a locally reduced concentration of glucosamine allowing the growth of cell lines with cytoplasmic G^R complements lower than what would normally be the threshold level. Such cell lines would possess higher G^R levels than the bulk of the background inoculum (thereby permitting establishment of a resistant microcolony), but on subculture and replating to GGM would still largely exhibit sensitivity to the full concentration of glucosamine in the medium (0.05%). Here again some transgessive cytoplasmically resistant cells would be able to overcome respiratory inhibition due to the locally reduced glucosamine content of the medium and estalbish microcolonies. The micorocolony response in this subsequent plating would presumably be greater than the original plating, and such is the case (c.f. 6/4BL, 6/4BLr, Fig. 5). A localized depletion of glucosamine is supported by observation of an inoculum effect for resistance on solid medium. It was found that GGM replica plates which had received heavier than usual inocula demonstrated greater resistance than could be attributed to transfer of cells with a resistance proportional to the growth increase. The greater inoculum, by greater uptake, may have reduce the locally available glucosamine to such a low level that cells with much lower than what is usually the threshold G^R content could proliferate in addition to normal microcolony cell lines. Consequently in the experiments reported here the GGM plate was always third or fourth in any series of plates replicated from one velvet pad.

In contrast, drug resistance is an all or nothing response. The process of inhibition does not consume the drug and it is therefore not surprising that G^R and E^R transmission exhibit different behaviours.

Another factor which must be considered in the attempt to understand the nature of the microcolony resistance response, and its relation to homogeneous resistance, is the intracellular basis of resistance. Strains or cell lines grown for prolonged periods in selective medium (GGM broth) will yield isolates of such high resistance that sensitive segregants are not detectable [10P3r, 6L4r, (4B2/10P3)r, and (4B2/6L4)r, Fig. 8 and Fig. 9]. Most dramatic of these is the history of 10P3r. This isolate was derived by glucosamine broth growth of inoculum from a resistant microcolony on a plate from the vegetative segregational analysis of 10P3, a glucosamine sensitive ascospore of the 10/4BL diploid (Fig. 8). Evidently even cell lines with such low intracellular G^R complements that they appear sensitive may be subjected to selection pressure to favour G^R determinants and sway the intracellular balance between G^R and G^S in favour of the former. The result of this intracellular selection pressure is establishment of stable and uniformly resistant cell lines. Does this selection pressure completely eliminate G^S determinants? Demonstration of the presence of G^S determinants in a mostly resistant population would be very difficult by growth selection on plates as described in this study. Microcolony sensitivity would be masked by the massive growth of resistant cells.

The intracellular threshold of G^R necessary in a cell for eliciting a glucosamine resistant response is the key to understanding the patterns of resistance observed in microcolony and highly resistant strains. While

the concept of a stable, mixed intracellular population of G^R and G^S is not consistent with the patterns observed for vegetative inheritance of E^R and E^S (THOMAS, 1969; RANK, 1970; BIRKY, 1973; LUKINS et al, 1973) it may confer an extremely advantageous form of glucosamine resistance upon cells. Erythromycin specifically and directly inhibits mitochondrial ribosomal functioning (CLARK-WALKER and LINNANE 1966). Sensitive mitochondria would rapidly be lost in the presence of erythromycin due to loss of functionality. The intracellular selection pressure favors E^R completely. In comparison, the action of glucosamine on mitochondrial function is probably only indirect and may vary in severity depending upon the concentration of inhibitor available (see preceding section). If cellular Pi depletion is responsible for glucosamine induced respiratory inhibition a critical balance between G^R and G^S within cells growing on glucosamine medium may be optimal for growth based on the following conjectures. Some or all cytoplasmic glucosamine resistance mutations affect the rate at which ATP is made available to cytosol via either production or transport. The mutant phenotype is that of a lower rate of ATP availability, and therefore a lower rate of energy dependent metabolism. If G^R is on mitochondrial DNA (discussed in next section) of which probably 50 to 100 molecules exist per cell (WILLIAMSON, 1969; DEUTSCH, DUJON, NETTER, PETROCHILO, SLONIMSKI, BOLOTIN-FUKUHARA and COEN, 1974) the total rate at which ATP is made available would be the sum of output by G^R and G^S containing mitochondria. If demonstration of the existence of only one, highly branched mitochondrion per cell by HOFFMAN and AVERS (1973) is accepted, mitochondrial ATP output would be a direct function of the G^R/G^S balance. Even if many mitochondria per cell exist this ratio would still determine mitochondrial ATP output.

Alteration of this G^R/G^S ratio may produce a whole range of sensitivity or resistance to glucosamine. For optimum growth at a particular glucosamine concentration a cell would require a G^R/G^S ratio which would incorporate the minimum G^R complement necessary to prevent severe Pi depletion, and the maximum G^S possible so as to least limit energy dependent metabolism, and therefore growth. Such a balance of G^R/G^S would obviously have a selective advantage over more sensitive ratios which would be growth inhibited, but more significantly the proper G^R/G^S level would allow cells to grow faster than a ratio higher in G^R and therefore more limited in energy metabolism.

The above model could account for the low resistance displayed by microcolonies which would accumulate only the minimum G^R to allow growth on solid medium. In liquid medium localized glucosamine depletion would be minimal requiring GGM broth grown cells to have a considerably higher G^R/G^S ratio. It is not possible to decide whether or not this enrichment process generates 100% G^R cell lines.

Location of G^R in GR10

The non-Mendelian nature of the G^R locus in GR10 was rather evident from the results of Phase I of this study. Microcolony resistance and vegetative segregation of the resistance response initially lead to this conclusion (Table I). Unequivocal confirmation for the non-Mendelian inheritance pattern of G^R in GR10 was gained from tetrad analysis of the 10/4BL backcross which exhibited tetrad classes including 1:3, 2:2, 3:1 and 0:4 (resistant:sensitive or r:s) all with resistant microcolonies (Table I). Demonstration that even sensitive spore isolates could vegetatively segregate resistant microcolonies reinforced the suspicion that cytoplasmic and not nuclear factors were involved in transmission of G^R in this strain.

The capacity of mitochondrial E^R mutations to become intracellularly predominant in cell lines placed under selection pressure has been quantified by BIRKY (1973). Under analagous selection pressure (plating to GGM) the sensitive spore isolate 10P3 (from 10/4BL) displayed some resistant microcolonies, one of which when subcultured into GGM broth (which placed it under severe selection pressure) yielded a very resistant isolate 10P3r (Fig. 6). To demonstrate the extent to which this intracellular selection could be invoked a further test was carried out. The enriched isolate, 10P3r was crossed to the parental 4B2, and a zygote clone was subcultured into liquid GGM. An isolate from this treatment (4B2/10P3)r exhibited dramatically increased levels of resistance over the analagous but unenriched backcross diploid 10/4BL (Fig. 6). No sensitive vegetative segregants were detected from this resistant diploid and 100% of the vegetative colonies showed confluent resistance. Tetrad analysis was even more emphatic. All tetrads exhibited 4:0 (R:S) spores [compare (4B2/10P3)r and 10/4BL in Table V] with each spore showing confluent resistance. Selective enrichability of the phenotypic response (glucosamine resistance) such as has just been described, along with the non-Mendelian inheritance pattern displayed in tetrad analysis constitutes very strong evidence for the cytoplasmic nature of this mutation.

If the basis of cytoplasmic glucosamine resistance in GR10 is a function coded by a cytoplasmic DNA such as mitochondrial DNA (mtDNA), then the loss of this DNA should result in a concomittant loss of glucosamine resistance. Ethidium bromide (EB) complexes with mtDNA to block mitochondrial transcription (GRIVELL and METZ, 1973) and produces an acid labile breakdown product, thus eliminating mtDNA (MAHLER and BASTOS, 1974). Ethidium bromide treatment of the highly resistant isolate 10P3r should therefore have produced a petite strain (10P3ρ^-) which possesed no

mtDNA and therefore was without the base sequences involved in glucosamine resistance or sensitivity (G°). Backcross of this G° strain to the G^S parental strain should then have resulted in only G^S determinants in all cells derived from zygotes.

Tetrad analysis of the diploid 4B2/10P3ρ^- failed to show any survival of G^R determinants in the EB petite 10P3ρ^-. All tetrads scored unfiormly as 0:4 (r:s) (Table V), as would be expected if G^R were a mutation on mtDNA. Vegetative segregational analysis also suggested that a dramatic loss of G^R determinants had been induced in EB treatment of 10P3r. Scoring of vegetative segregant clones showed that 98% were completely sensitive to glucosamine with the remaining 2% containing only one microcolony each (Fig. 8), also compatible with a mtDNA location for G^R in GR10 and its derivatives.

The reverse series of tests were also carried out to further substantiate the mtDNA location of G^R in GR10. Tetrad analysis of the EB derived rho$^-$ diploid 4B2ρ^-/10P3r demonstrated a definite increase in resistant spores per tetrad over the simple backcross (compare 4B2ρ^-/10P3r with 10/4BL in Table V. Analysis of vegetative segregant clones also showed an increase in resistance. The significant appearance of a number of confluently resistant clones was observed compared to the control 10/4BL (Fig. 8). However, neither the increase in vegetative resistance nor the shift toward greater resistance in tetrads was of the magnitude that one might predict. Failure of the cross to produce a diploid (4B2ρ^-/10P3r) of similar resistance to the contributing resistant haploid (10P3r) as was predicted may have been due to one or both of the following factors:

1. 4B2ρ^- may contain surviving G^S mtDNA
2. 10P3r may contain residual G^S determinants.

Though the EB treatment used to induce 4B2ρ^- as well as 10P3ρ^- and 6L4ρ^- was 100% efficient in petite induction it may not have been adequate to ensure complete elimination of mtDNA. Higher concentrations (10μg/ml) over equivalent treatment intervals have been employed with more success (SAUNDERS et al, 1971; BIRKY, 1973). It may therefore be true that there was some mtDNA survival in all three induced petites. This must have been minimal for 10P3ρ^-. Another possibility is that while the EB treatment employed was mostly effective at mtDNA elimination, the isolate 4B2ρ^- may have been an unfortunate choice containing surviving mtDNA.

The second of the above factors has already been disucssed at some length above. It is not possible to decide between these possibilities on the present evidence. Although the results are not as clear cut as was hoped, they still strongly support the hypothesis that G^R is a mitochondrially located mutation. Further evidence of a mtDNA location for G^R in GR10 would be evidence of an alteration in mtDNA in GR10 derivatives or demonstration of recombination between known mtDNA markers and G^R. The considerations above, it is felt constitute rather strong evidence in support of the conclusion that a mutation on mtDNA is the basis of glucosamine resistance in GR10. It has already been proposed that this locus be designated [CAT-1](ELLIOT and BALL, 1975). That this locus is different from known mitochondrial loci is supported by the lack of cross resistance reported in the results section.

Location of G^R in GR6

In preliminary analysis GR6 displayed much the same patterns of vegetative inheritance of glucosamine resistance as GR10 (Table I) and also exhibited similar patterns in tetrad analysis (Table III). These patterns were not compatible with classical Mendelian inheritance and therefore the same series of tests were carried out as for GR10.

As before, a heterogeneous tetrad was chosen from the backcross 64BL and each spore clone was subjected to vegetative segregational analysis. Of the three sensitive spores, two (6L1, 6L2 in Fig. 9) exhibited resistant microcolonies and one failed to show the presence of resistant determinants (6L3 in Fig. 9). The one spore isolate that had displayed microcolony resistance in tetrad analysis (6L4) contained resistant microcolonies in 71% of vegetatively segregated clones (Fig. 9). One microcolony from 6L4 was chosen and enriched from resistance in the manner described for 10P3r. This isolate 6L4r, showed confluent resistance in 100% of vegetative segregant clones (Fig. 9) indicating that intracellular resistance levels could be raised under selection pressure for resistant determinants. Selective enrichability of the G^R mutation in 6L4r was further investigated by its backcross to 4B2 and passage of the resultant diploid through GGM broth. An isolate from this sequence, (4B2/6L4)r showed greatly enriched resistance in vegetative segregational analysis (Fig. 9) with confluent resistance in all vegetative clones. Tetrad analysis was made difficult by low tetrad production in (4B2/6L4)r, and a high incidence of non-viable spores in tetrads (see Results Table VI) but all tetrads scored were 4:0, r:s with confluent resistance and no sensitivity detected. This evidence indicates that the G^R factor in GR6 behaves in the same manner as GR10 i.e. cytoplasmic inheritance.

The EB induced petite of 6L4r (6L4ρ^-) was backcrossed to 4B2 (cf. 10P3ρ^- backcross). In this case however, the diploid 4B2/6L4ρ^- rather than displaying loss or at least reduction of resistance levels over the simple backcross (6/4BL) displayed more resistant spores in tetrads (Table VI). Vegetative segregational analysis of 4B2/6L4ρ^- showed a similar pattern to 6/4BL with 99% of vegetative segregants containing one or more resistant microcolonies.

The results from the reverse cross (4B2 ρ^- X 6L4r) were very similar to those described for 4B2 ρ^-/10P3r. Tetrads showed more resistant spore responses that the simple backcross 6/4BL (compare 6/4BL and 4B2 ρ^-/6L4r in Table VI). Vegetative segregational analysis showed a similar pattern for 4B2 ρ^-/6L4r and 6/4BL with 100% of segregants containing microcolonies (Fig. 9).

The above behavior of the GR6 derivatives does not unequivocally support the contention that GR6 contains a mutation on mtDNA which confers glucosamine resistance. However, no concievable combination of Mendelian mutations could be considered compatible with the tetrad segregation patterns observed in these strains (Table VI). The most likely explanation of the anomalous patterns displayed by 4B2/6L4 ρ^- and 4B2 ρ^-/6L4r in tetrad and vegetative segregational analysis is that EB treatment was unsuccessful in eliminating all mtDNA in 6L4 ρ^- and 4B2 ρ^- as has already been discussed above. Partial elimination of mtDNA can lead to suppressive petite production (MEHROTA and MAHLER, 1968; NAGLEY and LINNANE, 1970; MICHAELIS, DOUGLAS, TSAI and CRIDDLE, 1971). If 6L4 ρ^- was a suppressive petite the anomalously high inheritance of G^R in the 4B2/6L4 ρ^- backcross would be explained. Similar results have been obtained by GINGOLD et al (1969) with a spontaneous petite of an E^R strain. Mutant GR6 and its derivatives do show the cytoplasmic inheritance patterns and intracellular selection under pressure characterisitc of cytoplasmic inheritance of altered mtDNA (RANK, 1970; BIRKY, 1973) which are not compatible with patterns expected from mutation of one or more Mendelian genes conferring glucosamine resistance.

Subsequent studies on 6L4r using 25 μg/ml of EB for 48 hr produced 6L4 ρ^- clones (5) which when backcrossed to 4B2 showed no retention of G^R at all (A.J.S. BALL personal communication) thus confirming the mitochondrial

location of G^R in strain GR6.

Recently, WAXMAN and EATON (1974) have suggested that the strain D587-4B, the original 4B2, possesses a nuclear gene controlling the transmission of mitochondrial DNA. If 6L4r did not contain this locus, 4B2 mitochondrial markers would act as suppressives in such crosses. This might also explain the behavior of 4B2ρ^-/6L4r and 4B2/6L4ρ^- (Fig.9, Table VI).

The Mendelian Locus gay-1

Occurence of only one nuclear glucosamine resistant mutant (GR133) in the group studied is not too surprising. Naked, cytoplasmic DNA's such as mtDNA are more suceptible to U.V. mutagenesis than nuclear DNA (MOUSTACCHI 1969). There was some degree of putative cytoplasmic resistance evident in GR133 as demonstrated by the microcolonies of 4B2/133 (Table II). Since expression of nuclear resistance was much faster (24 hr) than cytoplasmic resistance (72 hr minimum), a clear differentiation between the two responses was easy. It is clear from the tetrad analyses of the crosses 4B2 vs GR133 and aL1 vs 133F2 (Table II and IV) that the factor responsible for glucosamine resistance in GR133 is carried on nuclear DNA.

Because the mutations carried by aL1 only covered limited parts of linkage groups VII, IX and XI (see Appendix I), finding linkage to known loci in this cross ($133F_2$ vs aL1) would have been fortuitous indeed. Though some workers have been so blessed in other studies, the same grace did not prevail here. Other multi marker strains available at the time were either sterile or formed asporogenous zygotes with GR133 or 133F2. Due to such limitations linkage studies of the gay-1 locus were not pursued.

Status of G^R in the Balance of the Mutants

The discussion of Phase I above covers the entire group of mutants under study. However, in the light of the discussion of GR133, GR10 and GR6 some final considerations are in order at this point. The apparent conditional lethality exhibited by the strains GR120, GR124 and GR125 is of considerable interest in relation to the proposal of an optimum G^R/G^S balance in maintaining a heterogeneous cytoplasmic state in stable G^R mutants. It may well be that the 100% G^R state is lethal. Rather than an optimum G^R/G^S being merely advantageous perhaps it is an obligate state for survival on glucosamine medium. It may be then the G^R loci in these conditional lethal strains tend to purify themselves vegetatively, as do other mitochondrial drug resistant loci(eg. E^R; RANK, 1973; BIRKY, 1973), thus budding off lethal vegetative segregants.

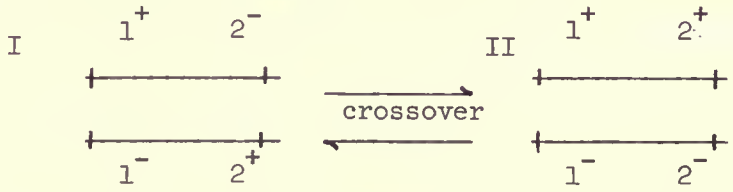
The cytoplasmic inheritance patterns of resistance displayed by strains GR5, GR8, GR9, GR22 and GR127 were similar to those of GR10 and GR6 (Table I, Table II, Table III). These mutations are most probably due to altered mtDNA. Mutants GR7, GR62 and GR112 exhibited rather anomalous growth characteristics and mating behavior which may or may not have been due to a G^R mutation. These strains, as well as the conditional lethals mentioned above were unlike the GR10 group (GR5, GR6, GR8 etc.) but still displayed clearly cytoplasmic inheritance patterns of resistance (Table I, Table II, Table III). On that basis they probably contain altered mtDNA which may or may not be allelic to [CAT-1].

Absence of a larger number of nuclear mutants in the group may not mean that most catabolite repression loci are on mtDNA but may reflect mutagen specificity (discussed above). Continuing characterization studies on other nuclear and cytoplasmic glucosamine resistant mutants not included here (Laboratory of Dr. A.J.S. BALL) should help indicate the number of

Mendelian and mitochondrial functions involved. Subsequent elucidation of the operational relationships of such mutants should certainly contribute toward an integrated understanding of mitochondriogenesis.

ADDENDUM

It has been suggested by Dr. Sherman that some variety of mitotic recombination phenomenon (MORTIMER AND HAWTHORNE, 1969) might explain the vegetative segregation phenomena observed for all strains excepting GR133. A similar result was obtained for mikamycin resistance by Linnane et al who falsely concluded that vegetative segregation was sufficient evidence for for cytoplasmic inheritance[1]. These authors started by isolating mutants in a diploid strain, a practice not followed here (see METHODS). The mechanism invoked is as follows:



if in this situation 1 and 2 are alleles of the same locus, and $1^{+}2^{-}$ and $1^{-}2^{+}$ are inactive forms of the locus, the equilibrium will exist between I (resistant) and II (wild type or sensitive). During meiosis one might observe 2:2 (r:s) segregation (from II) or 4:0, 1:3 or 2:2 (from I). One difference between this phenomena and true cytoplasmic inheritance is that if one isolates 4:0 (r:s) tetrads one should not be able to isolate 0:4 (r:s) tetrads (from I). This explanation can only be used to explain segregation in the haploid strains (Tables I and II) by postulating a chromosome duplication followed by successive mutations to give alleles 1^{-} and 2^{-} (see above) in the GR strains. In general, mitotic recombination events and/or chromosome loss in aneuploids occur at a relatively constant rate for any one locus or any one chromosome pair (MORTIMER AND HAWTHORNE, 1969). One would not expect to see the large variations in segregation rates within strains or between strains if this

explanation was true, unless one invokes several different GR loci all of which (co-incidentally) were subjected to chromosome duplication in the haploid form.

If GR6 and GR10 were aneuploids carrying two different GR loci, then the occurence of vegetative segregation in the diploids could be explained (although not its variability) and also the enrichment for GR isolates in GGM broth. However, this phenomena cannot account for the simultaneous observations of 0:4, 1:3, 2:2, 3:1 and 4:0 tetrads (Table III).

Similarly the role of EB and the ρ^- mutation in reducing either G^R or or G^S input/output in diploids (Figures 8 and 9) is not explained by the mitotic recombination theory. This result is much more compatible with cytoplasmic, and particularly mitochondrial inheritance.

Although it may be possible to construct more complicated nuclear models, involving multiple copies of nuclear genes and/or chromosomes to explain the observed data, the most parsimonious explanation which readily explains all of the observed data for GR6 and GR10 is that of cytoplasmic inheritance.

Lastly, one must acknowledge that the persistence of mixed (or apparently mixed) G^R/G^S clones through many diploid generations is not typical of the drug resistance markers C^R, E^R, O^R and P^R (LINNANE, HOWELL AND LUKINS, 1974) studied by other workers. We have no explanation to offer, other than that postulated in the body of the thesis i.e. some form of positive selection for G^R/G^S mixtures.

1. LINNANE, A.W., C.L. BUNN, N. HOWELL, P.L. MOLLOY and H.B. LUKINS, 1975. The phenomenology of cytoplasmic genetics in yeast: A proposal for an autonomy of mitochondrial membranes and the determinism of nucleo-cytoplasmic genetic interactions, in Biochemistry of Gene Expression in Higher Organisms, J.K. Polluk and W. Lee eds. pp 425-442. Australian and New Zealand Book Co. Ltd., New South Wales.

Literature Cited

Acs, G. and F.B. Straub 1954. Concerning the metabolism of ascites tumor cells. Doklady Acad. Nauk SSSR 95;1021-1024

Ainsworth, P.S., R.M. Janki, E.R. Tustanoff and A.J.S. Ball 1974. Incorporation of cytochrome oxidase into micochondrial membranes. Bioenergetics 6;135-150.

Aloni, Y. and G. Attardi 1971. Experession of the mitochondrial genome in HeLa cells.II Evidence for complete transcription of mitochondrial DNA. J. Mol. Biol. 55;251-266.

Avner, P.R. and D.E. Griffiths 1970. Oligomicin resistant mutants in yeast. FEBS Letters 10;202-207.

Avner, P.R. and D.E. Griffiths 1973. Studies on energy linked reactions. Genetic anlaysis of oligomycin-resistant mutants of Saccharomyces cerevisiae. Eur. J. Biochem. 32;312-321.

Avner, P.R., D. Coen, B. Dujon, and P.P. Slonimski 1973. Mitochondrial genetics IV. Allelism and mapping studies of oligomycin resistant mutants in S. cerevisiae. Mol. gen. Genet. 125;9-52.

Ball, A.J.S. and E.R. Tustanoff 1970. Effect of D(-)- and L(+)-threo-chloramphenicol and nucleotide and related respiratory activities in yeast undergoing metabolic repression and de-repression. Biochim. Biophys. Acta 199;476-489.

Beck, J.C., J.H. Parker, W.X. Balcavage and J.R. Mattoon 1971. Mendelian genes affecting development and function of yeast mitochondria. in Autonomy and Biogenesis of Mitochondria and Chloroplasts. N.K. Boardman, A. W. Linnane and R. M. Smillie, eds. American Elsevier, N.Y. pp 194-204.

Belitzer, W.A. 1936. Uber die umgekehrte Pasteursch Reaktion. Biochem. Z. 283;339-342.

Bessell, E.M. and P. Thomas 1973. The deoxyfluoro-D-glucopyranose 6-phosphates and their effects on yeast glucose phosphate isomerase. Biochem. J. 131;77-82.

Biely, P., Z. Kratky and S. Baur 1974. Metabolism of 2-deoxy-D-glucose by bakers yeast VI. A study on cell wall mannan. Biochim. Biophys. Acta 353;268-274.

Birky, C. W. 1973. On the origin of mitochondrial mutants: Evidence for intracellular selection of mitochondra in the origin of antibiotic-resistant cells in yeast. Genetics 74;421-432.

Boardman, N.K., A.W. Linnane and R.M. Smillie 1971. eds, Autonomy and Biogenesis of Mitochondria and Chloroplasts. American Elsevier, N.Y.

Bolotin, M., D. Coen, J. Deutsch, B. Dujon, P. Netter, E. Petrochilo and P.P. Slonimski 1971. La recombinason des mitochondries chez Saccharomyces cerevisiae. Bulletin de L'institut Pasteur 69;215-239.

Brin, M. and R.W. McKee 1956. The inhibition of respiration by glucose, fructose and mannose in Ehrlich mouse ascites tumor. Cancer Res. 16;364-368

Bunn, C.L., C.H. Mitchell, H.B. Lukins and A.W. Linnane 1970. Biogenesis of mitochondria XVII. A new class of cytoplasmically determined antibiotic resistant mutants of Saacharomyces cerevisiae. Proc. Nat. Acad. Sci. U.S. 67;1233-1240.

Burk, D. 1939. A colloquial consideration of the Pasteur and neo-Pasteur effects. Cold Spring Harbour Symp. Quant. Biol. 7;420-459.

Chance, B. and B. Hess 1956. On the control of metabolism in ascites tumor cell suspensions. Ann. N.Y. Acad. Sci. 63;1008-1016.

Chance, B. and B. Hess 1959. Spectroscopic evidence of metabolic control. Science 129;700-708.

Chapman, C. and W. Bartley 1968. The kinetics of enzyme changes in yeast under conditions that cause the loss of mitochondria. Biochem. J. 107;455-465.

Chen, S.Y., B. Ephrussi and H. Hottinguer 1950. Nature genetique des mutants a defecience respiratoire de la souch B-II de la levure de boulangerie. Heredity 4;337-351.

Cirillo, U.P. 1962. Mechanism of glucose transport accross the yeast cell membrane. J. Bact. 84;485-491.

Clark-Walker, G.D. and G.L.G. Miklos 1974. Mitochondrial genetics, circular DNA and the mechanism of the petite mutation in yeast. Genetical Res. 24;43-57.

Crabtree, H.G. 1929. Observations on the metabolism of tumors. Biochem. J. 23;536-545.

Coen, D., J. Deutsch, P. Netter, E. Petrochilo, and P.P. Slonimski 1970. In Control of Organelle Development. Soc. Exp. Biol. Symp. 24;449-492, P. Miller ed. Cambridge University Press, London.

Deutsch, J., B. Dujon, P. Netter, E. Petrochillo, P. P. Slonimski, M. Bolotin-Fukahara, and D. Coen 1974. Mitochondrial Genetics VI. The petite mutation in Saccharomyces cerevisiae: Interpretations between the loss of the ρ^- factor and the loss of drug resistance mitochondrial genetic markers. Genetics 76;195-219.

Ebner, E., L. Mennucci and G. Schatz 1973. Mitochondrial assembly in respiration-deficient mutants of Saccharomyces cerevisiae I. Effects of nuclear mutations on mitochondrial protien synthesis. J. Biol. Chem. 248;5360-5368.

Ebner, E. and G. Schatz 1973. Mitochondrial assembly in respiration-deficient mutants of Saccharomyces cerevisiae III. A nuclear mutant lacking mitochondrial adenosine triphosphatase. J.Biol. Chem. 248;5379-5384.

Elliot, J.J. and A.J.S. Ball 1973. Glucosamine resistance in the yeast Saccharomyces cerevisiae. Genetics 74;S71.

Elliot, J.J. and A.J.S. Ball 1974. A preliminary analysis of glucosamine resistance in yeast. Proc. Can. Fed. Biol. Soc. 17;50.

Elliot, J.J. and A.J.S. Ball 1975. A new mitochondrial mutation in yeast Biochem. Biophys. Res. Comm. 64;277-281.

Emmelot, P. and C.J. Bos 1959. The metabolism of neoplastic tissue. The effect of 2:4-dinitrophenol on the respiration of ascites tumor cells, Brit. J. Cancer 13;520-550.

Ephrussi, B. and H. Hottinguer 1950. Direct demonstration of the mutagenic action of euflavin on Baker's yeast. Nature 166;956.

Ephrussi, B. and P.P. Slonimski 1950. La synthese adaptave de cytochromes chez la levure de boulangerie. Biochim. Biophys. Acta. 6;256-267.

Ephrussi, B. and H. Hottinguer 1951. Cytoplasmic constituents of heredity. On an unstable state in yeast. Cold Spring Harbour Symp. Quant. Biol. 16;75-85.

Ephrussi, B., H. de Margerie - Hottinguer and H. Roman 1965. Supressiveness: A new factor in the genetic determination of the synthesis of respiratory enzymes in yeast. Proc. Nat. Acad. Sci. US 41; 1065-1071.

Ernster, and Drahota 1969. Mitochondria: Structure and Function. Academic Press, London.

Errington, P. and A.J.S. Ball 1974. The inhibition of respiration by glucosamine in yeast. Proc. Fed. Can. Biol. Soc. 17;51.

Fogel, S. and R.K. Mortimer 1971. Recombination in yeast. Ann. Rev. Genetics 5;219-236.

Gatt, S. and E. Racker 1959. Regulatory mechanisms of carbohydrate metabolism I. Crabtree effect in restructed systems. J. Biol. Chem. 243;1015-1023.

Gingold, E.B., G.W. Saunders, H.B. Lukins, and A.W. Linnane 1969. Reassortment of the cytoplasmic genetic determinants for respiratory competence and erythromycin resistance. Genetics 62;735-744.

Griffiths, D.E., R.L. Houghton and W.E. Lancashire 1974. Mitochondrial genes and ATP-syntetase. in The Biogenesis of Mitochondria. A.M. Kroon and C.Saccone, eds. Academic Press, N.Y. pp 215-223.

Groot, G.S.P., L. Kovac and G. Schatz 1971. Promitochondria of anaerobically grown yeast. V. Energy transfer in the absence of an electron transfer chain. Proc. Nat. Acad. Sci. US 68; 308-311.

Hansen, and O. Winge 1935. Compt. rend. trav. lab. Carlsberg, Ser. physiol. cited by R.K. Mortimer and D.C. Hawthorne, 1969. Yeast genetics. in The Yeasts. A. Rose and J.S. Harrison eds. Academic Press, London, pp385-460.

Hartwell, L.H. 1970. Biochemical genetics of yeast. Ann. Rev. Genetics 4;373-396.

Haslam, J.M., M. Perkins and A.W. Linnane 1973. Biogenesis of mitochondria 32. A requirement for mitochondiral protein synthesis for the normal adenine nucleotide transporter in yeast mitochondria. Biochem. J. 134;935-947.

Hawthorne, D.C. and R.K. Mortimer 1968. Genetic mapping of nonsense suppressors in yeast. Genetics 60;735-742.

Hess, B. and B. Chance 1959. Phosphorylation efficiency of the intact cell. I. Glucose-oxygen titrations in ascites tumor cells. J. Biol. Chem. 234;3031-3035.

Hess, B. and B. Chance 1961. Metabolic control mechanisms VI. Chemical events after glucose additon to ascites tumor cells. J.Biol. Chem. 236;239-246.

Hoffman, H-P. and C.J. Avers 1973. Mitochondrion of yeast: Ultra-structural evidence for one giant, branched organelle per cell. Science 181;749-751.

Howell, N., P.L. Molloy, A.W. Linnane and H.B. Lukins 1974. Biogenesis of mitochondria 34. The synergistic interaction of nuclear and mitochondiral mutations to produce resistance to high levels of mikamycin in Saccharomyces cerevisiae. Mol. gen. Genet. 128;43-54.

Ibsen, K.H., E.L. Coe and R.W. McKee 1958. Interrelationship of metabolic pathways in Ehrlich ascites carcinoma cells. I. Glycolysis and respiration (Crabtree effect). Biochem. Biophys. Acta 30;384-400.

Ibsen, K.H. 1961. The Crabtree effect: A review. Cancer Res. 21;829-841.

Jacob, F. and J. Monod 1961. Regulatory mechanisms in the synthesis of proteins. J..Mol. Biol. 3;318-356.

James, A. P. and Spencer 1958. The process of spontaneous extra nuclear mutation in yeast, Genetics 43;317-331.

Jayaraman, J., C. Cotman, H.R. Mahler and C.W. Sharp 1966. Biochemical correlates of respiratory deficiency VII. Glucose repression. Arch. Biochem Biophys. 116;224-251.

Jennings, D.H. 1974. Sugar transport into fungi: An essay. Trans. Brit. Mycol. Soc. 62;1-24.

Johnston, J.R. and R.K. Mortimer 1959. Use of snail digestive juice in isolation of yeast spore tetrads. Genetics 78;292.

Keller, F.A. and E. Cabib 1971. Chitin and yeast budding. Properties of chitin synthetase from Saccaromyces carlsbergensis. J. Biol. Chem. 246;160-166.

Krebs, H.A. 1972. in Essays in Biochemistry. P.N. Campbell and F. Dickens eds. Academic Press, N.Y., vol 8, ppl.

Kroon, A.M. and C. Saccone 1974. The Biogenesis of Mitochondria. Academic Press, N.Y.

Kotyk, A. 1965. Properties of the sugar carrier in baker's yeast I. Kinetics of transport. Folia Microbiologica 10: Cited by KOTYK,(1973).

Kotyk, A. 1967. Properties of the sugar carrier in baker's yeast II. Specificity of transport. Folia Microbologica 12: Cited by KOTYK, (1973).

Kotyk, A. and D. Michaljanicova 1968. Properties of the sugar carrier in baker's yeast IV. An assymetric component of monosaccharide transport. Folia Microbiologica 13: Cited by KOTYK (1973).

Kotyk, A. 1973. Molecular aspects of non-electrolyte transport in yeasts. Proc. 3rd Int. Special Symp. on Yeasts. Otaniemi/ Helsinki p 103-132.

Kovac, L. and Weissova 1968. Oxidative phosphorylation in yeast III. ATPase activity of the mitochondrial faction from a cytoplasmic respiratory-deficient mutant. Biochem. Biophys. Acta 153;55-59.

Kovac, L. 1974. Biochemical mutants: An approach to mitochondrial energy coupling. Biochem. Biophys. Acta 346;101-135.

Lederberg, J. and E.M. Lederberg 1952. Replica plating and indirect selection of bacterial mutants. J. Bact. 63;399-406.

Letansky, K. 1968. Strain variations in utilization of hexoses by Ehrlich ascites tumor cells. Biochem. Biophys. Acta 165;364-373.

Linnane, A. W., G. W. Saunders, E. B. Gingold and H. B. Lukins 1968. The biogenesis of mitochondria V. Cytoplasmic inheritance of erythromycin resistance in Saccharomyces cerevisiae. Proc. Nat. Acad. Sci. U.S. 59; 903-910.

Linnane, A. W. and J.M. Haslam 1970. The biogenesis of yeast mitochondria. Curr. Topics in Cellular Metab. 2;101-171.

Linnane, A.W., N. Howell and H.B. Lukins 1974. Mitochondrial genetics. in Biogenesis of Mitochondria. A.M. Kroon and C. Saccone eds. Academic Press, N.Y. pp 193-213.

Loomis, W.F. and F. Lipman 1948. Reversable inhibition of coupling between phosphoylation and oxidation. J.Biol. Chem. 173;807-808.

Lukins, H.B., J.R. Tate, G.W. Saunders and A.W. Linnane 1973. The biogenesis of mitochondria 26. Mitochondrial recombination: The segregation of parental and recombinant mitochondrial genotypes during vegetative division of yeast. Mol. gen. Genet. 102;17-25.

Lyen, F., G. Hartmann, K. F. Netter and A. Schuegraf 1959. Phosphate, turnover and Pasteur effect. CIBA Foundation Symp. or Regulation of Cell Metab. G.E.W. Wolstenholm & C.M. O'Connor eds. Churchill, London. pp 256-276.

Mahler, H.R. and R.N. Bastos 1974. Coupling between mitochondrial mutation and energy transduction. Proc. Nat. Acad. Sci. U.S. 71;2241-2245.

Mehrota, B.D. and H.R. Mahler 1968. Characterization of some unusual DNA;s from the mitochondria of certain "petite" strains of Saccharomyces cerevisiae. Arch. Biochem. Biophys. 128;683-703.

Michaelis, G., S. Douglas, M. J. Tsai and R. S. Criddle 1971. Mitochondrial DNA and supressiveness of petite mutants in Saccharomyces cerevisiae. Biochem. Genet. 5;487-495.

Michels, C.A., J. Blamire, J. Goldfinger and J. Marmur 1974. A genetic and biochemical analysis of petite mutations in yeast. J. Mol. Biol. 90;431-450.

Miller, P., ed. 1970. Control of Organelle Development. Soc. Exp. Biol. Symp. 24. Cambridge Univ. Press. London.

Mitchell, C. H., C.L. Bunn, H.B. Lukins and A.W. Linnane 1972. Biogenesis of mitochondria 23. The biochemical and genetic characteristics of two different oligomycin resistant mutants of Saccharomyces cerevisiae under the influence of cytoplasmic genetic modification. Bioenergetics 4;363-379.

Molloy, P.L., N. Howell, D.T. Plummer, A.W. Linnane and H.B. Lukins 1973. Mitochondrial mutants of yeast Saccharomyces cerevisiae showing resistance in vitro to chloramphenicol inhibition of mitochondrial protein synthesis. Biochem. Biophys. Res. Comm. 52;9-14.

Montencourt, B.S., S-C Kuo and J.O. Lampen 1973. Saccharomyces mutants with invertase formation resistant to repression by hexoses. J.Bact. 114;223-238.

Mortimer, R.K. and D.C. Hawthorne 1966. Yeast genetics. Ann. Rev. Microbiol. 20;151-168.

Mortimer, R.K. and D.C. Hawthorne 1969. Yeast genetics. in The Yeasts I. A. Rose and J.S. Harrison eds. Academic Press, N.Y. pp 385-460.

Mortimer, R.K. and D.C. Hawthorne 1973. Genetic mapping in Saccharomyces. IV. Mapping of temperature-sensitive genes and use of disomic strains in localising genes. Genetics 74;33-54.

Moustacchi, E. 1969. Cytoplasmic and nuclear genetic events induced by UV light in strains of Saccharomyces cerevisiae with different UV sensitivities. Mutation Res. 7;171-185.

Nagley, P. and A.W. Linnane 1970. Mitochondrial DNA deficient mutation of yeast. Biochem. Biophys. Res. Comm. 39;989-996.

Perlman, R., B. Chen, B. Decrombrugghe, M. Emmer, M. Gottesman, H. Varmus and I. Pastan 1970. The regulation of lac operon transcription by cyclic adenosine 3', 5' monophosphate. Cold Spring Harbour Symp. Quant. Biol. 35;419-423.

Perlman, P.S. and C.W. Birky 1974. Mitochondrial genetics in baker's yeast: A molecular mechanism for recombinational polarity and supressiveness. Proc. Nat. Acad. Sci. U.S. 71;4612-4616.

Perlman, P.S. and H.R. Mahler 1974. Depression of mitochondria and their enzymes in yeast: Regulatory aspects. Arch. Biochem. Biophys. 162;248-271.

Perkins, M., J. M. Haslam, H. R. Klyce and A.W. Linnane, Bongkrekic acid resistant mutants of Saccharomyces cerevisiae. FEBS Letters 36; 137-142.

Pittman, D.D. 1959. Ultraviolet induction of respiration deficient varients of Saccharomyces and their stability during vegetative growth. Cytologia 24;315.

Polakis, E.W., W. Bartley and G.A. Meek 1964. Changes in the structure and enzyme activity of Saccharomyces cerevisiae in response to changes in the environment. Biochem. J. 90;369-374.

Polakis, E.S. and W. Bartley 1965. Changes in the enzyme activities of Saccharomyces cerevisiae during aerobic growth on different carbon sources. Biochem. J. 97;284-301.

Polakis, E.S., W. Bartly and G.A. Meek 1965. Changes in the activities of respiratory enzymes during the aerobic growth of yeast on different carbon sources. Biochem. J. 97;298-302.

Polakis, E.S. and W. Bartley 1966. Changes in the intracellular concentrations of adenosine phosphates and nicotinimide nucleotides during the aerobic growth cycle of yeast on different carbon sources. Biochem. J. 99;521-533.

Racker, E. 1956. Carbohydrate metabolism in ascites tumor cells. Ann. N.Y. Acad. Sci. 63;1017-1021.

Racker, E. 1974. History of the Pasteur effect and its pathobiology. Mol. Cellular Biochem. 5;17-23.

Rank, G.H. 1970. Genetic evidence for "Darwinian" selection at the molecular level I. The effect of the supressive factor on cytoplasmically-inherited erythromycin resistance in Saccharomyces cerevisiae. Can. J. Genet. Cytol. 12;129-136.

Rank, G.H. and N.T. Bech-Hansen 1972. Somatic segregation, recombination, assymetrical distribution and complementation tests of cytoplasmically-inherited antibiotic-resistance mitochondrial markers in S. cerevisiae. Genetics 72;1-15

Reilly, C. and F. Sherman 1965. Glucose repression of cytochrome a synthesis in cytochrome-deficient mutants of yeast. Biochem. Biophys. Acta 95;650-651.

Renner, E.D., P.G.W. Plageman and R.W. Bernlohr 1972. Permeation of glucose by simple and facilitated diffusion by Novikoff rat hepatoma cells in suspension culture and its relationship to glucose metabolism. J. Biol. Chem. 247;5765-5776.

Roodyn, D.B. and D. Wilkie 1968. The Biogenesis of Mitochondria Methuen, London.

Sanders, H.K., P.A. Mied, M. Briquet, J. Hernandez-Rodriquez, R.F. Gottal, and J.R. Mattoon 1973. Regulation of mitochondrial biogenesis: Yeast mutants deficient in synthesis of δ-aminolevulinic acid. J. Mol. Biol. 80;17-39.

Saunders, G.W., E.B. Gingold, M.K. Tremblath, H.B. Lukins and A.W. Linnane 1971. Mitochondrial genetics in yeast: Segregation of a cytoplasmic determinant in crosses and its loss or tetention in petite. in Autonomy and Biogenesis of Mitochondria and Chloroplasts. N.K. Boardman, A.W. Linnane and R.M. Smillie eds. North Holland, N.Y. pp 185-193.

Schatz, G. 1968. Impaired binding of mitochondrial adenosine triphosphatase in cytoplasmic "petite" mutant of Saccharomyces cerevisiae. J.Biol. Chem. 243;2192-2199.

Schatz, G. and Mason 1974. The biosynthesis of mitochondrial protiens. Ann. Rev. Biochem. 43;51-87.

Sherman, F. 1959. The effects of elevated temperatures on yeast II. Induction of respiratory-deficient mutants. J. Cell Comp. Physiol. 54;37-52.

Sherman, F. 1963. Respiration deficient mutants in yeast I. Genetics 48;357-385.

Sherman, F. 1964. Mutants of yeast deficient in cytochrome c. Genetics 49;39-48.

Sherman, F. and P.P. Slonimski 1964. Respiration deficient mutants of yeast II. Biochemistry. Biochem. Biophys. Acta 90;1-15.

Sherman, F. and J.W. Stewart 1971. Genetics and biosynthesis of cytochrome c. Ann. Rev. Genet. 5;257-296.

Slonimski, P.P., G. Perrodin and J. Croft 1968. Ethidium bromide induced mutation of yeast mitochondria: Complete transformation of cells into respiratory deficient non-chromosomal "petites". Biochem. Biophys. Res. Comm. 30;232-239.

Souverijn, J.H.M., L.A. Huisman, J. Rosing and A. Kemp jr. 1973. Comparison of ADP and ATP as substrates for adenine nucleotide translocator in rat liver mitochonria. Biochem. Biophys. Acta 305;185-198.

Thomas, D.Y. and D. Wilkie 1968a Inhibiton of mitochondrial synthesis in yeast by erythromycin: Cytoplasmic and nuclear factors controlling resistance. Genet. Res. 11;33-41.

Thomas, D.Y. and D. Wilkie 1968b. Recombination of mitochondrial drug resistance factors in Saccharomyces cerevisiae. Biochem. Biophys. Res. Comm. 30;368-372.

Thomas, D.Y. 1969. Mitochondrial mutants in yeast. Ph. D. Thesis, cited by BIRKY, 1973.

Tiedemann, H. 1952. Uber den Stoffwechsel des Ascites Tumors der Maus. Atschr. ges. exper. Med. 119;272-279.

Tustanoff A, and W. Bartley 1964. Development of respiration in yeast grown anaerobically on different carbon sources. Biochem. J. 91;595-600.

Tzagloff, A. and P. Megher 1972. Assembly of the mitochondrial membrane system VI. Mitochondrial synthesis of subunit proteins of the rutamycin-sensitive adenosine triphosphatase. J. Biol. Chem. 247;594-603.

Utter, M.F., E.A. Duell and C. Bernofski 1968. in Aspects of Yeast Metabolism. A.K. Mills ed. Blackwell, Oxford. pp 197-205.

Van Steveninck, J. and A. Rothstein 1965. Sugar transport and metal binding in yeast. J. Gen. Physiol. 49;235-246.

Van Steveninck, J. 1968. Transport and transport-associated phosphorylation of 2 deoxy-D-glucose by yeast. Biochem. Biophys. Acta 136;386-394.

Van Steveninck, J. 1969. The mechanism of transmembrane glucose transport in yeast: Evidence for phosphorylation associated with transport. Arch. Biochem. Biophys. 130;244-262.

Waxman, M.F. and N.R. Eaton 1974. Nuclear factors and the control of supressivness in petite mutants of Saccharomyces cerevisiae. Mol Gen. Genet. 133;37-45.

Williamson, D.H. 1969. The effect of environmental and genetic factors on the replication of mitochondrial DNA in yeast. in Soc. Exp. Biol. Symp., E.P. Miller ed. Cambridge Univ. Press. London. pp 71-84.

Winge, O. and O. Lausten 1939. Compt. Rend. trav. lab. Carlsberg, Ser. physiol. 22;357. cited by MORTIMER and HAWTHORNE, 1969.

Wu, R. and E. Racker 1959. Regulatory mechanisms in carbohydrate metabolism IV. Pasteur effect and Crabtree effect in ascites tumor cells. J. Biol. Chem. 234;1036-1041.

Ycas, M. and T.J. Starr 1953. The effect of glycine and protoporphyrin on cytochrone deficient yeast. J. Bact. 65;83-88.

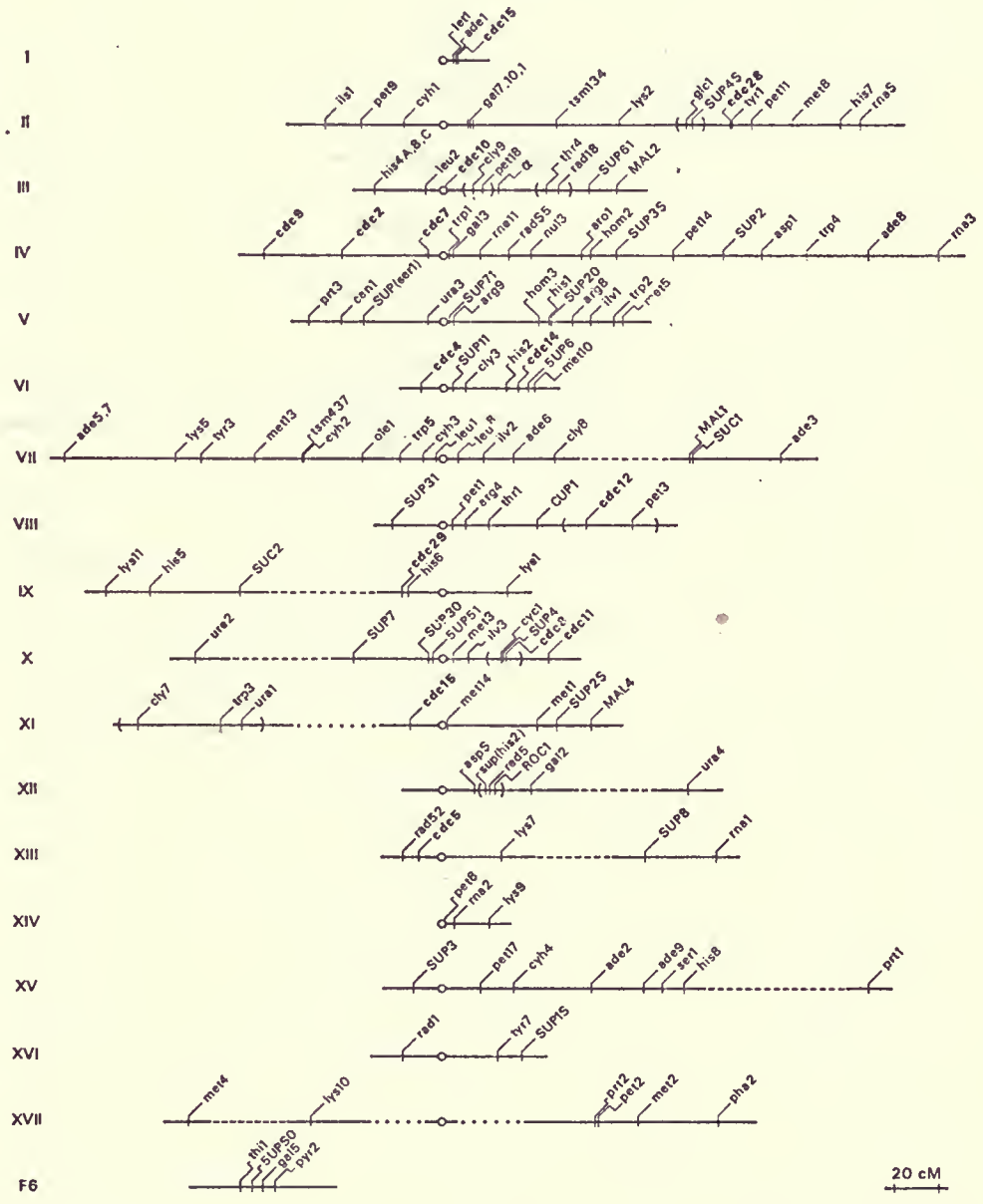
Appendix I (a). Linkage Map of Saccharomyces*

(b). Linkage Map of Saccharomyces Showing Markers carried by aL1.

* from MORTIMER and HAWTHORNE (1973).

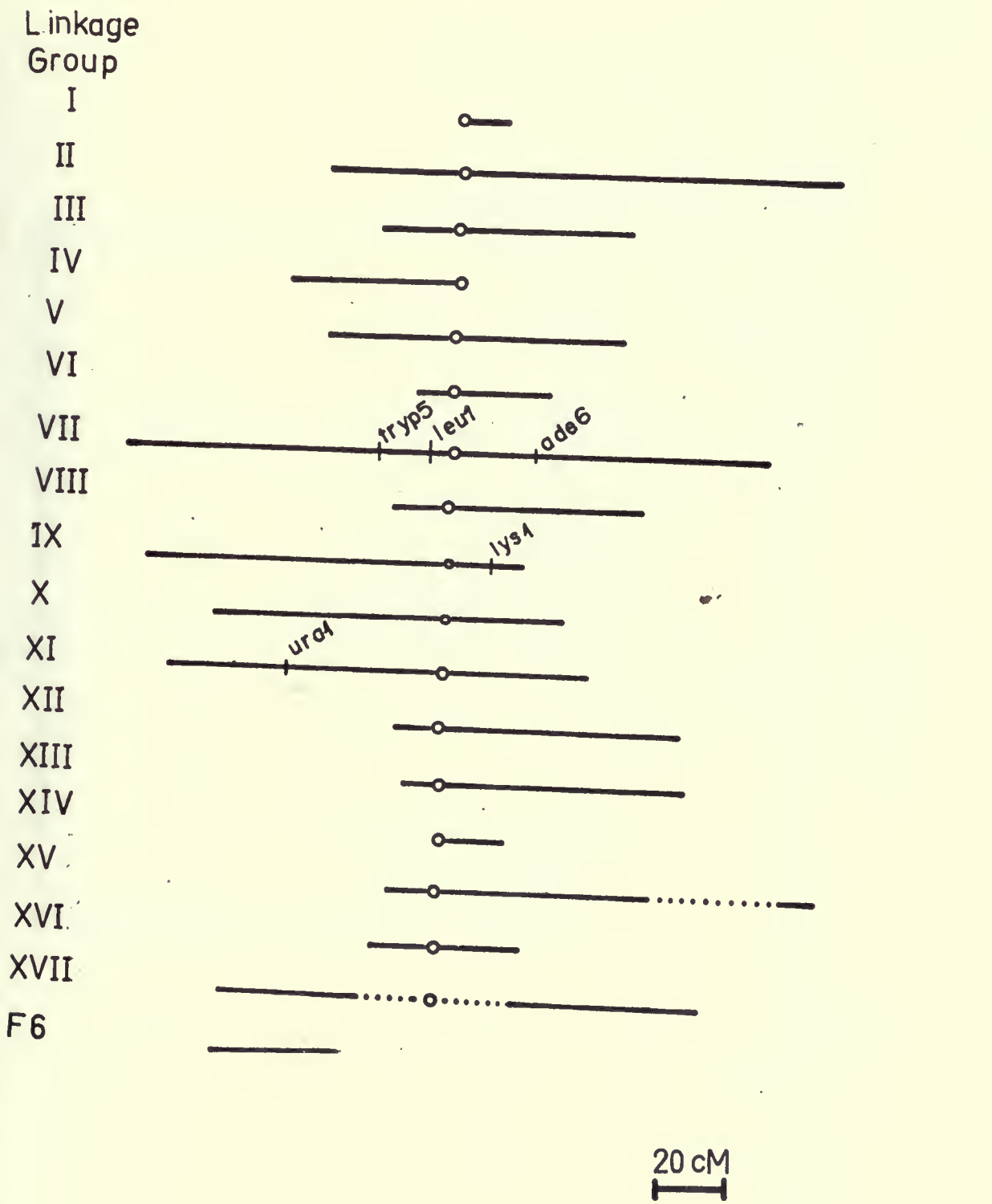
Appendix I(a)

Linkage Map of Saccharomyces



Appendix I(b)

Linkage Map of Saccharomyces



Appendix II. Proposed Mitochondrial Gene Maps

All loci represent drug resistant mutation.

a) OL1 = oligomycin resistance
ERY = erythromyan resistance
CAP = chloramphenical resistance
MIK = mikamycin resistance
SPI = spiramycin resistance

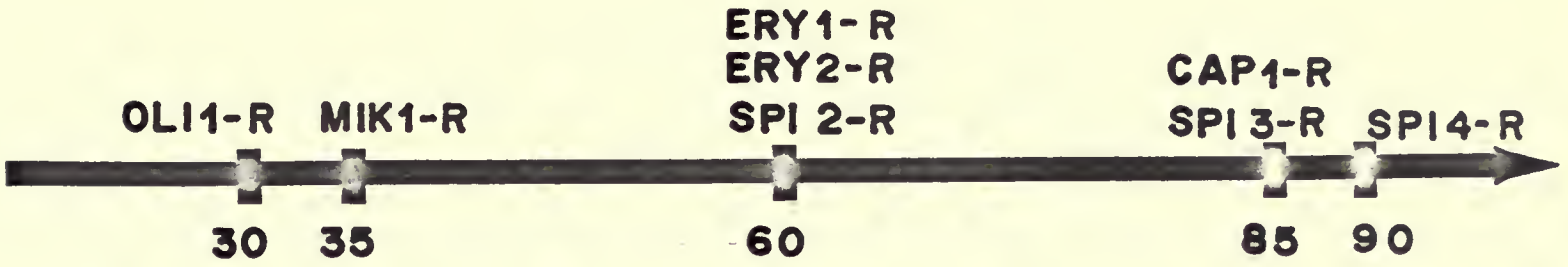
from LINNANE, HOWELL and LUKINS, 1974

b) R_I, R_{II}, R_{III} = mutations affecting ribosome functions and are equivalent to 60, 85 and 90 in (a).
O_I is equivalent to 30, 35 in (a)
O_{II} is the second oligomycin resistant gene
T = triethyltin resistance
V = venturicidin resistance
P = rho factor

broken lines indicate the presence of unliked loci on the same mtDNA.

from GRIFFITHS, HOUGHTON and LANCASIRE, 1974

Appendix II (a)



(b)