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A STUDY OF THE EFFECTS OF ELEVATED
TEMPERATURES ON THE GROWTH AND
INHERITANCE OF *SACCHAROMYCES CEREVISIAE*

BERKELEY, CALIFORNIA

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UCRL-8573
Biology and Medicine

UNIVERSITY OF CALIFORNIA

Lawrence Radiation Laboratory
Berkeley, California

Contract No., W-7405-eng-48

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(Thesis)

November 1958

Printed for the U.S. Atomic Energy Commission

Printed in USA. Price \$1.75. Available from the
Office of Technical Services
U. S. Department of Commerce
Washington 25, D. C.

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ABSTRACT

A comparative study was made of the growth of yeast in various media at the optimum temperature (30°) and at supraoptimum temperatures. It was found that at elevated temperatures there is a decrease in the ability of yeast to grow, which may be alleviated by (a) increasing the percentage of yeast extract in the medium, (b) adding oleic acid to the medium, or (c) using an inoculum of cells that have previously been grown at the elevated temperature. Because of these findings, it is believed that growth at elevated temperatures results in an increased nutrient requirement which may be eliminated by induced adaptation.

When yeasts were grown at elevated temperatures or exposed for a short time to lethal temperatures it was found that there was a great increase in the fraction of respiratory-deficient mutants (petites). It was shown that the increase of mutants did not arise because of selection, but that the elevated temperatures actually induced the mutation. From the results of various genetic analyses it was shown that these respiratory deficient mutants are very similar, if not identical, to vegetative petites occurring spontaneously or induced by acriflavine. The kinetics of this mutation is discussed, with possible theoretical interpretations.

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I. INTRODUCTION

The use of temperature as a parameter to elucidate biological processes is most salient. This is not surprising, since most, if not all, biochemical reaction rates are temperature-dependent. With this in mind one may interpret a temperature optimum of a biological process as a result of at least two reactions: "one accelerating, the other diminishing, the process, and both increasing, though to different extents, with rise in temperature."⁷⁵ The decrease with supraoptimum temperatures is assumed to be the result of thermal destruction or inhibition of enzymatic activity, and therefore it is of great interest to study biological processes in this temperature region.

The investigation presented here was initiated primarily for the study of the production of respiratory-deficient mutants (petites) in yeast by growth at elevated temperatures and by heat shock. However, during the course of the investigation an increased nutrient requirement for growth at elevated temperatures was noted, which was also studied. Preliminary results of the former effect have been reported by Ycas²⁰⁶ and the author.¹⁶²

The increase of nutrient requirements for growth at supraoptimum temperatures has been reported for a number of microorganisms (see general review by Precht, Christophersen, and Hensel,¹³⁶ pp. 107, 192, 264). The most probable explanation of this phenomenon is the inactivation or inhibition of the requisite enzymes at these higher incubation temperatures. Evidence for this hypothesis is available from the study by Maas and Davis,¹⁰⁴ who have shown that the enzyme synthesizing pantothenic acid in a mutant of E. coli, which required the vitamin only at temperatures above 30°, was extremely thermolabile, compared to the enzyme of the wild type, which was not dependent on pantothenic acid. Horowitz and co-workers^{69, 57} have also found that tyrosinase of Neurospora crassa can occur in two forms which differ in thermostability.

Since this paper deals in part with respiratory-deficient mutants it is appropriate to consider details of some of the previous work in this area. Respiratory-deficient yeast arises spontaneously from normal cells and under most conditions constitutes approximately 1% of a growing population. Such mutants are easily detected by their different colony

morphology, being smooth, white, and also smaller when plated on solid nutrient medium in which glucose is a growth-limiting factor. These "vegetative petite" mutants have been extensively studied by Ephrussi and co-workers and by others (for recent general reviews see Ephrussi, 39, 40 Wright, 201 and Winge and Roberts 195), who have shown them to be cytoplasmic mutants probably arising from an irreversible loss or inactivation of self-reproducing cytoplasmic units which are necessary for the synthesis of cytochrome oxidase.

Besides the absence of cytochrome oxidase, vegetative petites also lack cytochromes a and b and possibly other enzymes, 167, 98 which results in an almost complete loss of respiration, and an inability to utilize certain substrates as a carbon source, 147, 126, 203, 204 but an unimpaired fermentation. 166 The enzymes that are lacking in the vegetative petites are linked in the normal strain to particles, sedimentable by centrifugation, which it is thought may be mitochondria. 167 Furthermore, the mitochondria of the petite mutants, which are morphologically similar to those of the normal strain, are unstainable with janus green, indicating a loss of cytochrome c-oxidase. 48, 78

Similar types of mutations have been reported to occur in Neurospora crassa, 27 in Candida albicans, 2 and possibly in higher plants. 199, 200, 115, 116

Proof of non-Mendelian inheritance of vegetative petites was furnished by genetic analysis of diploid progeny resulting from a cross of petite and normal haploid strains. 44 Such diploid zygotes produce phenotypically normal cells, as did the cultures derived from the four spores of a single ascus. Successive backcrosses of the spores with the parental petite also resulted in evidence favoring a non-Mendelian interpretation. Even more direct confirmation of the cytoplasmically inherited characteristics of vegetative petites was furnished by examination of bud clones from isolated heterokaryons, which were found to have changes in respiratory phenotype not associated with recombination of nuclear markers. 201, 202

Recent work of Ephrussi, Margerie-Hottinguer, and Roman has shown that there are two classes of vegetative petites, neutral (as described above) and suppressive. 46 This latter type differs from the neutral petite in that it elicits a petite phenotype when crossed with a normal cell. Petite strains, however, can have a variety of degrees of "suppressiveness" ranging from 0 to nearly 100% i. e., per cent of zygotes giving rise to mutant clones. A decrease in suppressiveness can occur spontaneously or can be experimentally induced. Wright has shown that the degree of suppressiveness undergoes variation during vegetative growth. 201

Besides the vegetative petites, there exist phenotypically similar petite mutants which are a consequence of single-gene mutations, 29, 146, 147 and therefore show Mendelian segregation when crossed with normal strains.

When the two types of petites are crossed the resulting diploids are normal, indicating that the "segregational" petite contains cytoplasmic factors that the vegetative petite lacks. Furthermore, it has been shown tentatively that the vegetative petites lack the apoenzymes, and the segregational petites lack the prosthetic groups of the cytochromes.⁴⁰

The rate of mutation to the vegetative petites can be enhanced by a number of chemical and physical agents. The most extensively studied are certain acridines, which are highly active and specific mutagens. Ephrussi and co-workers have shown that a yeast population grown for 48 hours in the presence of acriflavine is composed almost entirely of petite cells, although in the absence of proliferation no effect was observed.^{43,45} It was further shown by the use of microtechniques that the phenomenon is due directly to a mutagenic action and not to selection.^{41,42} Furthermore, this high mutation rate is somewhat preserved after the cells have been removed from the acriflavine.⁴² By using euflavine, Marcovitch has shown that the mutation rate can be very close if not equal to unity, i. e., all buds formed in the presence of the dye are mutants.¹¹¹ He has also shown that the mutagenic and toxic effects of euflavine can be attributed to different properties of the molecule.^{112,113} This mutagenic property of euflavine can be alleviated by certain agents.¹⁶⁹ In a recent paper Nagai and Nagai have shown that the induction of petites with acriflavine can occur in a medium in which the petites cannot proliferate.¹²³

It is of interest to note that acriflavine has also been reported to induce "cytoplasmic" mutations in trypanosomes,^{188,71} Bodo caudatus¹⁵⁰ Aspergillus nidulans,⁷ and Escherichia coli.⁶⁸

Vegetative petites can also be induced with great efficiency by growth in triphenyltetrazolium chloride,⁸⁷ p-nitrophenol,^{203,204} propamidine isothionate,⁹⁴ and manganous salts.^{97,124} Copper, cobalt, and nickel salts also induce petites, but are extremely lethal.⁹⁷ Other chemical agents have been reported to produce respiratory-deficient yeast, although the exact nature of these variants was not reported. Some of these agents are ethylene oxide,¹⁸⁹ hydrogen cyanide,¹⁷⁶ sodium azide, crystal violet, janus black and other agents,^{192,193,194} camphor,⁵⁶ and styryl.¹⁰⁸

Raut has shown that ultraviolet irradiation can increase the frequency of petites to more than 50% of the survivors, the bulk of these petites being vegetative mutants.¹⁴⁷ The action spectrum of this phenomenon indicated that it is due to the absorption by nucleic acid.¹⁴⁸ Raut and Simpson also found that x-irradiation has only a slight effect on petite production.¹³² Pittman has studied the efficiency of inducing petites with uv irradiation of haploid and tetraploid strains and concludes that the induction frequency is essentially ploidy-independent.¹³² By analyzing uv mutation-dose-response curves, he also concluded that the number of self-reproducing particles inactivated in order to produce a vegetative petite is in the neighborhood of five per cell.¹³³ Sarachek has reported the photoreactivation of uv-induced petites in cultures adapted

to aerobic respiration; no effect of photoreactivation was observed in unadapted yeast.¹⁵⁴ The accurate uv dose-response curves of Elkind and Sutton (in press) indicate that petite cells are being produced from two different populations.³⁷ A second rise in the petite-production curve is correlated with the uv-resistant portion of the total survival curve.

Using a haploid strain of yeast, Lindegren and Hino have reported that anaerobic growth causes a tenfold increase in petite variants over the frequency under aerobiosis, which could not be accounted for through selection or dispersal of cell clusters.⁹⁵ However, Harris⁶⁵ and Sarachek¹⁵⁴ found that the rate of mutation to petites was independent of oxygen during growth of the diploid strains they employed.

Ephrussi and Hottinguer have reported a strain which has a high rate of spontaneous mutation to vegetative petites because of a single genetic locus.⁴² Other unstable strains have been reported by James and Spencer⁷³ and Gutz.⁶³

Thus the mutation rate has been shown to be strongly influenced by chemical, physical, and genetic factors. In this investigation it is shown that temperature also has a pronounced effect which can help elucidate the nature of the petite variant.

II. MATERIALS AND METHODS

A. Yeast Cultures

The six different genetically marked strains of Saccharomyces cerevisiae used in this investigation were obtained from Dr. Robert K. Mortimer. These were three haploid cultures, AS3, S1796A, and S288C, and three diploid cultures, X320, X495, and X674, with the following genotypes:

AS3	a	Su ₁	ma	g ₂	his ₄
S1796A	a	Su ₁	ma	g ₂	tr ₅
S288C	a	Su ₁	ma	g ₂	
X320	a/a	me ₁ /+	tr ₁ /+	ur ₁ /+	
X495	a/a	me ₁ /+	tr ₁ /+	ur ₁ /+	his ₄ /+ ad ₆ /+
		thr ₂ /+	leu ₁ /+		
X674	a/a	me ₁ /+	ur ₁ /+	tr ₂ /+	his ₁ /+ ad ₁ /+
					his ₄ /+

The above symbols designate: mating types (a or a); nutrient requirements of histidine (his), leucine (leu), tryptophane (tr), threonine (thr), uracil (ur), methionine (me), adenine (ad); inability to ferment galactose (g), and maltose (ma); ability to ferment sucrose (Su). The subscripts refer to allelic designation.¹¹⁹

When petite variants of a strain were desired, the culture was plated and a spontaneously occurring petite colony was isolated. Unless stated otherwise the petite strains were transferred seven to eight times on Preinoculation medium, with 24 hours of growth between transfers in order to stabilize them in growth rate.⁶⁵ The designation of such a derived petite strain is the strain number with a suffix "S."

B. Preparation of Growth Media

In most cases, liquid growth media (YED) contained 4% dextrose, 0.5% KH_2PO_4 and Bacto-yeast extract.

Yeast-extract solutions were sterilized by filtration, and dextrose and buffer solutions were sterilized by autoclaving separately for 15 minutes at 120°C. Solutions were stored at 4°C and mixed before using. This latter procedure was found necessary for obtaining reproducible results when growth at elevated temperatures was being studied. For example, no growth at 40°C could be obtained if a solution of 6% YED (6% yeast extract, 4% dextrose, 0.5% KH_2PO_4) had been stored at room temperature for a month.

Plating medium (PL), for the determination of viability, containing 0.5% yeast extract, 0.5% dextrose, and 2% agar, was sterilized by autoclaving. Preinoculation medium (PI) containing 0.5% yeast extract, 1% dextrose, and 2% agar was similarly prepared.

Bacto-potato-dextrose agar (PD) was prepared as directed. Bacto-potato-dextrose broth, which is identical to the former medium less agar, was specially ordered from Difco Laboratories.

C. Growth Curves

In most experiments yeast was grown in 10 ml of medium placed in 50-ml flasks covered with beakers, and shaken in a Dubnoff metabolic shaking incubator (Precision Scientific). When this procedure was used there was no evaporation even after several weeks of incubation. Unless stated otherwise the culture flasks were inoculated with 10^5 cells/ml of yeast which were previously grown for 24 hours on PI medium at 30°C. Under these conditions the inoculum consisted of about 98% single cells.

In the analysis of cell number, several criteria may be employed (see, e. g., Morris, ¹¹⁸). Turbidimetric methods, the most frequently employed technique, were not used in this study on account of the variability of culture media with respect to transmission and turbidity, the difficulty of comparing viable counts and cell counts, and the change of cell size with different temperatures. Cell numbers were determined in these studies by (a) hemocytometer chamber count of cells plus buds, (b) hemocytometer count of clusters, (c) viable count by dilution plating method. Therefore, in the determination of a growth curve as a function of time, aliquots were withdrawn from the culture flask at suitable intervals and analyzed by hemocytometer chamber count and by streaking a diluted volume containing about 100 viable cells on the surfaces of plates of PL medium. The streaked plates were incubated at 30°C for about 4 days for viable counting and assay of petite frequency. At this time there was no ambiguity in the differentiation of normal and petite colonies. In this investigation all mixed or "scallop" colonies⁴² were scored as normal.

D. Single-Cell Analyses

The analyses of single cells were conducted by methods similar to those of Ephrussi and Hottinguer,^{41,42} Spiegelman, DeLorenzo, and Campbell,¹⁷³ and Spiegelman,¹⁷² and consisted in isolating the progeny of single yeast cells by microtechniques.

The procedure used in this study was as follows: A cover slip was coated with 6% YED and 2% agar and then placed in a moist chamber. Twenty-four-hour PI-grown cells were streaked on a corner of the nutrient agar slab, and single cells were isolated by use of a microneedle (for description of the micromanipulator and microneedle see Burns.²³) After budding had occurred the desired cell was moved to a convenient location, where it was removed by a method of Fowell,⁵³ which consists of marking the coverslip above the single cell with a drop of ink, removing the coverslip from the moist chamber, and cutting the agar portion immediately below the marking. This entire procedure was conducted in a temperature-regulated box at $40.5 \pm 0.5^\circ\text{C}$.

The agar block which contained the single cell was then placed in a test tube containing 0.5 ml of 6% YED and 0.5 ml of water, and incubated at 30°C . If growth occurred after 5 days of incubation the tube was shaken and either a diluted aliquot or a loop of cell suspension was plated on PL medium.

E. Heat Shock

The cultures used in the preliminary heat-shock experiments were prepared by spreading a dilute suspension of yeast on either PI or PD medium and incubating at 30°C . After the desired time, cells were harvested off the surface of the plates and suspended. Survival curves were obtained as follows: The suspension of yeast was diluted to about 10^6 cells/ml in sterile distilled water at the desired inactivation temperature ($54 \pm 0.01^\circ\text{C}$) and immediately agitated. Aliquots were withdrawn as a function of time and plated on PL medium.

The same procedure was employed for later heat-shock experiments except that the cultures were prepared by growth at 30°C in liquid PD medium for 3 days, and instead of water, 0.5% KH_2PO_4 was used in the inactivation solution.

The plates inoculated with the heat-treated cells were incubated for 4 or 5 days at 30°C , after which time colony counts were made. Unlike the growth experiments, these showed a spectrum of colony sizes. For this reason there was difficulty in differentiating the normal and petite colonies, and the "tetrazolium overlay technique" was employed.¹²⁷

F. Genetic Analyses

The mating of cells was performed by the mass-mating technique,⁹⁶ i. e. by mixing a loopful of the two cultures on PI medium. Eight hours after the crosses were made the mass-mating mixtures consisted mainly of zygotes and the parental haploid cells. At this time the suspension was plated on synthetic media in which only the prototrophic zygotes were able to proliferate.

Sporulation was induced by transferring 2-day cells grown in presporulation medium (2% Difco nutrient agar, 1% yeast extract, 5% dextrose, 2% agar) to sporulation medium (0.3% sodium acetate, 0.02% raffinose, 2% agar) and incubating at 25°C for 1 week.¹ Four-spore asci were then isolated and dissected by microtechnique.

III. RESULTS

A. Nutrient Requirements for Growth at Elevated Temperature

The growth of normal and petite strains in various amounts of yeast extract at 30°C, the optimum-growth-rate temperature, is shown in Fig. 1. Although the growth of the normal strain is relatively independent of the percentage of yeast extract, the petite strain shows a marked decrease in growth rate in 0.5% YED. Such decrease of growth rate is usually associated with the absence of a partial requirement in the medium.^{82, 118}

When growth is studied at a higher temperature (40°C) many quantitative and qualitative differences are observed. One interesting difference is that after 1 day of incubation of the normal yeast at 40°C, more than 99% of the cells of the resultant population are petite. This phenomenon is elaborated in detail in the next section.

Another important difference is the great dependence of growth of normal and petite yeast on the amount of yeast extract (Figs. 2, 3, 4). In 0.5% YED growth proceeds through only a few divisions, with a drop in viability. Growth in 1% YED proceeds similarly to that in 0.5% YED except that the initial death phase is only temporary and an increase of viable cells is observed after 2 days (Fig. 3B). In higher percentages of yeast extract, growth is relatively rapid. It should be noted that although the maximum titer of cells in stationary phase is independent of the percentage of yeast extract, the number of cells per cluster was found to decrease with increasing amounts of yeast extract. The death phase occurring after stationary phase was found to be rather pronounced at 40°C; no death was observed at 30°C even after 1 week of incubation.

Fig. 1. Growth of X495 (normal diploid) and X495-S (petite diploid) at 30°C in 4% dextrose, 0.5% KH_2PO_4 , and various amounts of yeast extract. N refers to the number of cells plus buds.

MU-14702

Fig. 2. Growth of X495 at 40°C in various percentages of YED. N refers to the number of clusters.

MU-14705

Fig. 3. Growth of X495-S at 40°C in YED of the following percentages: A, 0.5% B, 1% C, 2% D, 6% E, 8%. $\circ$ refers to cells plus buds; $\bullet$ to clusters; Δ to viable count. F indicates growth in 0.5% YED in which the inoculum consisted of cells grown for 8 days in 1% YED at 40°C (curve B).

MU-14703

Fig. 4. The number of days for the viable count to reach 10^6 /ml when yeast is grown in various amounts of YED at 40°C . A is X495-S (petite); B is X495 (normal). X refers to the acclimatized yeast (see Fig. 3F).

Attempts to grow yeast in synthetic medium (Bacto Yeast Nitrogen Base, 1% dextrose) at 40°C were unsuccessful, and the resulting growth curves were similar to those of 0.5% YED.

If, after 2 weeks' incubation at 40°C the 0.5% YED culture flask is transferred to 30°C, growth proceeds rapidly to a titer comparable to that of Fig. 1. This indicates that the elevated temperature does not affect the growth medium and that the inhibition of growth is highly reversible. This is illustrated schematically in the top of Fig. 5. Also, if cells grown at elevated temperatures were used for the inoculum, growth could be initiated in 0.5% YED. This is illustrated in Fig. 3F, in which the inoculant was yeast which had been grown for 8 days at 40°C in 1% YED (Fig. 3B). Furthermore, this latter effect can be lost if the elevated-temperature-grown cells are transferred and grown at 30°C before inoculation in 0.5% YED. This is schematically represented in Fig. 5, illustrating the reversibility of this adaptation process.

There are undoubtedly many nutrients that affect the growth characteristics in limited amounts of yeast extract at elevated temperatures. Although no extensive investigation of this problem was undertaken, it was noted that ethanol solutions of oleic acid can greatly influence the growth curve in 1% YED at 40°C (Figs. 6, 7). As seen in Fig. 6E, the initial death phase can be entirely eliminated by the addition of 1.0 μ mole/ml of oleic acid. Addition of oleic acid in 0.5% YED, however, could not initiate growth.

B. Production of Petites by Growth at Elevated Temperatures

As previously mentioned, growth of normal yeast at elevated temperatures resulted in an increase in the fraction of petite variants, as ascertained by observing the colony forms (Fig. 8). Besides the normal and petite colonies many "scalloped" or revertant colonies, containing mixtures of normal and petite cells, were also observed. This is also found in induction with acriflavine.⁴²

Quantitative results of this phenomenon were studied by growing various strains of yeast at different temperatures in 6% YED (Figs. 9 and 10). It can be seen that growth at these elevated temperatures results in an enormous shift in the population to predominantly petite cells. However, the corresponding growth curves of the petite strains (Figs. 11, 3D) indicate that they have a longer generation time, about 1.5 times as long at 38°C as for a culture inoculated with primarily normal cells.

It is of interest to note the difference of petite production in the strain X495, which has a higher fraction of petites when grown at 30°C--10% in the steady state when grown in 6% YED, compared with 1% and 3% of strains X320 and X674 respectively. Also, it should be noted that temperature has less effect in the production of petites in the haploid strains.

MU-14771

Fig. 5. A schematic representation of various experiments (see text). Stippling indicates growth.

Fig. 6. Growth of X495-S at 40°C in oleic acid. A, 1% YED (control); 1% YED plus 1% ethanol with various amounts of oleic acid; B, 0; C, 0.01; D, 0.1; E, 1.0; F, 2.0 μmole/ml. ○ refers to cells plus buds; ● to clusters; Δ to viable count.

MU-14704

Fig. 7. The number of days for the viable count to reach 10^6 /ml when X495-S is grown in 1% YED at 40°C in various amounts of oleic acid.

ZN-2119

Fig. 8. Petri plates showing a spontaneously occurring petite colony (upper left); mixture of petite and normal colonies after 10 hours of growth at 40°C (upper right); after 24 hours, showing petite colonies (lower left), and normal colonies growing over background of petite cells (lower right).

MU-14969

Fig. 9. Growth of various diploid strains at various temperatures in 6% YED. ○ refers to number of cells plus buds; □ to viable count of normals; ● to petites. Dashed line refers to the total viable count.

MU-15142

Fig. 10. (Above) Fraction of normals after 24 hours of growth with various diploid (X674, X320, X495) and haploid (AS3, S1796A) strains as a function of temperature. (Below) Total viable count after the same period of growth.

MTL-15183

Fig. 11. Growth at 38°C of various petite strains in 6% YED. Cells plus buds O ; clusters ● ; viable Δ .

C. Single-Cell Analyses

Although the shapes of the growth curves and the extreme efficiency of elevated temperatures in increasing the level of petites seem to be indicative of induction and not selection, this evidence is not conclusive. Direct proof of induction and its underlying mechanism is furnished by the results of the single-cell analysis.

In this investigation two lineages at 40.5°C were considered:

(a) The daughter series, in which a yeast cell was kept at the elevated temperature until the first bud could be removed. At this time the mother cell was placed at 30°C and the bud retained at 40.5°C until it budded, and the process was then repeated.

(b) The sister series, in which a yeast cell was retained at the elevated temperature and its first, second, etc. buds removed for incubation at 30°C. After the last bud was removed, and the sister series terminated, the mother cell was also returned to 30°C.

At no time was there any confusion in the differentiation between a mother and a daughter cell, mainly because at this temperature the mother cell always budded prior to its daughter, as previously reported by Burns for 38°C.²³

The results of these experiments are given in Table I and schematically illustrated in Fig. 12. The daughter series clearly proves that the growth at elevated temperatures actually induces the mutation and that it is not merely the ability of petites to grow at a faster rate, i.e., selection. This is consistent with the results of the mass-culture technique. The data obtained indicate that the probability is very low for the original mother cell to produce petite buds. However, the first-generation cells produce about one-half petite buds. Whether or not the original normal cells can be induced to the petite phenotype could not be determined because of the high degree of inviability when they are transferred to 30°C.

Because of the method of the above study, a cell was considered normal when it gave rise to a clone containing as little as 0.1% normals. In order to determine the degree of heterogeneity, the resulting progeny of the single cells were plated out after the culture had grown to stationary phase (about 25 generations). The results of the first experiment of each series are shown in Table II and seem to indicate that a high mutation rate was preserved over many cell generations. It should be remarked that with the method of cultivation of the single cells by a standing culture the percentage of petites is higher than by a shaking culture (aerated), being 10% and 1% respectively in a control study. It should also be remembered that under this condition of considerable growth, selection plays an important role, and if any normal cells were present, the culture would tend to approach a steady-state value of 10% petites. Therefore, if sampling were performed earlier, before many generations could have occurred, the fraction of petite variants was greatly enhanced.

Results of single-cell analyses at 40.5°C (see text)

Results of single-cell analyses at 40.5°C(see text)										
	Generations									
	0	1	2	3	4	5	6	7	8	
Daughter series	+	+	+	-	-	Δ				
	+	+	Δ	+	-	-				
	+	+	Δ	+	Δ	-				
	Δ	Δ	-	-	-	-				
	+	+	+	-	Δ	-	Δ			
	+	+	+	Δ	-	-				
	+	+	Δ	Δ	-	-	-			
	+	+	-	-	-					
Sister series	+	+	+	+	+	+	+			
	Δ	+	Δ	Δ	Δ					
	Δ	+	+	+	+	+	+			
	Δ	+	+	+	+	+				
	Δ	+	+	+	+	+	+	+	+	+
	Δ	+	+	+	+	+	+	Δ	+	+
	Δ	+	+	+	+	+	Δ	+	+	+
	Δ	+	+							
	Δ	+	+	+	+	Δ	-	+		
	Δ	+	+	-	Δ	+	-	-		

+ = normal
 Δ = inviable

MU-15,357

Fig. 12. Schematic representation of Table I.

Table II

Per cent of petites resulting when single cells are taken from 40.5°C and allowed to proliferate at 30°C (see text)

	Generations					
	0	1	2	3	4	5
Daughter series	26.3	30.5	79.0	100	100	
Sister series	16.2	18.3	19.7	21.8	23.1	20.0
Control	10.5					

In this investigation some interesting side observations indicated that elevated temperatures could induce many abnormal cell types. When log-phase cultures were employed as inoculants, many filamentous cells were observed similar to the types induced by other agents.¹¹⁸ Also, several dumbbell-shaped cells were observed which appeared identical to previously reported ultraviolet-induced types.^{181, 156} These anomalous cell types were not observed with the liquid mass-culture technique.

D. Heat Shock and Induction of Petites

An important question is whether the rate of mutation to the petite variant can be enhanced in nonproliferating cultures exposed to elevated temperatures. The result of such an experiment is shown in Fig. 13. In this experiment a 24-hour culture of 10^5 cells/ml was exposed to 40°C in the absence of yeast extract, with and without dextrose. Curves A and D of Fig. 13 show that in the absence of dextrose the viability drops only to about 50% in 3 days, with no significant change in the fraction of petites. However, in the presence of dextrose there is a marked drop in viability and a corresponding increase in the percentage of petites (Fig. 13, B and C). This latter case may be similar to the previous experiments, in which low concentrations of yeast extract were employed in the growth medium (Figs. 2, 0.5%, 3A), and although little growth took place there was a marked increase in the number of petites. It should also be remembered that at these temperatures autolysis takes place,⁷⁷ and in the presence of dextrose there is probably a rapid turnover of yeast cells. One may therefore say that the marked drop in viability and the increase in the fraction of petites are occurring in a "growing" culture.

Fig. 13. Heat inactivation at 40°C of X674 (normal diploid):
A; in 0.5% KH_2PO_4 ; D, with the corresponding percentage
of petites; B and C, in 0.5% KH_2PO_4 plus 4% dextrose.

The conclusion that nonproliferating cultures exposed to elevated temperatures do not increase their yield of petite mutants was further exemplified when yeast was exposed to an above-maximum-growth temperature, 45°C. It was found that there was no increase in the fraction of petites, even in the presence of yeast extract, although a drop in viability occurred. This seems to indicate the independence of petite production and cell death.

However, when the temperature of exposure is markedly increased, death proceeds rapidly and a high percentage of survivors of the heat inactivation develops into petite colonies. Before going into details of this observation one should make several remarks on variations of sensitivity to lethal temperatures. Preliminary results of several experiments at 54°C (Fig. 14) indicate that:

- (a) culture age had a great effect on survival curves, older cultures being more resistant;
- (b) the type of medium in which the cells were incubated prior to the heat exposure greatly influenced the survival curve, cells grown in PD (potato dextrose agar) being more resistant than cells grown in PI (0.5% yeast extract, 1% dextrose, agar);
- (c) an isolated petite variant X320-S was more sensitive to heat shock than the normal strain;
- (d) haploid yeast cells are more resistant than cells of related diploid strains, and higher ploidies are even more sensitive;
- (e) multicomponent survival curves occurred which could not be explained by clusters, buds, sporulation, or differential drying.

The petite strain X320-S was found to be unstable with respect to its heat sensitivity, for it became more resistant during storage at 4°C. It differed from other petite strains in anomalous growth curves (at 30°C and higher temperatures; see Fig. 11) and in giving a lower maximum titer. Another marked difference of this strain was that heat-treated cells showed fewer survivors when plated on synthetic medium than on yeast-extract medium. For the above reasons the strain was abandoned and a second petite strain was isolated, X320-S2. The second petite strain was much more resistant to heat shock than the original petite isolate, but it should be pointed out that in both cases the petite strains were always more sensitive than the related normal strains.

Figure 15 shows the results of inactivating 3-day PD-grown cells at 54°C, and also the corresponding increase in the percentage of petites. Included for comparison is the survival curve of X320-S2-1, a petite variant which was transferred once in PI medium. From the results of the above experiments it must be concluded that the increase of petite mutants did not arise from selection, but was induced by the heat shock, since (a) petite strains are more sensitive to the heat treatment, and (b) there is an increase in the absolute number of petite colonies after a short exposure to heat. This latter point is illustrated by considering the results of the 10-minute exposure. The survivors of this treatment showed more than ten times as great an absolute number of petite colonies as in the original inoculum.

Fig. 14. Heat inactivation at 54°C of X320 (normal diploid): A, grown 1 day on PI; B, 14 days on PI; C, 16 days on PD; H, of S288C (normal haploid) grown 16 days on PD; P, of X320-S (petite diploid) grown 3 days on PI.

Fig. 15. Heat inactivation at 54°C of X320 (normal diploid): A, grown 3 days on liquid PD; C, with the corresponding percentages of petites; B is a similarly treated petite strain (X320-S2-1).

Examination of Fig. 15 reveals the similarity of the petite survival curve to the tail of the normal survival curve. This similarity is illustrated in Fig. 16, in which the axis of the graph has been shifted to allow coincidence of the curves. Also included in Fig. 16 is a survival curve of X320-S2-15, the petite strain after it has been transferred 15 times on PI medium, allowing 24 hours between transfers. From this comparison one may say that the petite strains respond in much the same way as a population of normal cells that have survived a period of heat exposure.

E. Genetic Analyses

Several genetic tests were undertaken in order to determine the similarity of petites obtained by different methods. For this study several petite variants were isolated, from two haploid cultures of opposite mating type (AS3, S1796A), that had arisen by (a) spontaneous occurrence of petites (-S), (b) growth for 24 hours in 3% YED and 5 mg/l acriflavine at 30°C (-AC), (c) growth for 24 hours in 6% YED at 40°C (see Fig. 10) (-H), (d) heat shock at 54°C (-T).

Preliminary spectroscopic examinations of the differently obtained petite strains indicated that there were no obvious differences.

When these petite variants were crossed in all combinations (e.g., AS3-S1 X S1796A - H1, spontaneously occurring petites with petites obtained by growth at 40°C) the resulting zygotes produced only petite cells. Since it has been previously shown that spontaneous and acriflavine-induced petites are usually vegetative mutants, the conclusion is that these differently obtained petites were not supplemented by each other and segregational petites were not induced.

The degree of suppressiveness⁴⁶ was obtained by crossing the various petites with the normal strain of opposite mating type and measuring the percentage of zygotes giving rise to petite clones (Table III). The data are insufficient to determine whether or not one method of induction produces a significant difference in the amount of suppressive petites.

The cross S1796A X AS3-H1 (normal X 40°C-induced petite) was sporulated and several four-spore asci were dissected; 2:2 segregation of the histidine and tryptophane markers was observed, and all spores gave rise to normal clones, as would be expected for crosses involving vegetative petites.

From the results of these genetic analyses one may conclude that the petites obtained by heat shock and growth at elevated temperatures are very similar, if not identical, to the vegetative petites occurring spontaneously or induced by acriflavine. All methods of induction produce some suppressive petites.

Fig. 16. Heat inactivation at 54°C of various normal and petite strains of X320. (Note that the axes have been shifted to allow coincidence of the curves.)

Table III

Suppressiveness of differently obtained petites. The per cent suppression is measured by the percentage of zygotes giving rise to petite clones minus the percentage of spontaneous petites in the normal strain, which is 6.8% for AS3 and 2.1% for S1796A.

Origin of petite	normal	X	petite	% petites	% Suppression
Spontaneous	AS3	X	S1796A-S1	32.5	25.7
	AS3	X	S1796A-S2	72.3	65.5
	S1796A	X	AS3-S1	58.4	56.3
	S1796A	X	AS3-S2	82.3	80.2
Acriflavine	AS3	X	S1796A-AC1	1.9	~0
	AS3	X	S1796A-AC2	94.0	87.2
	S1796A	X	AS3-AC1	2.2	~0
	S1796A	X	AS3-AC2	1.1	~0
Growth at 40°C	AS3	X	S1796A-H1	48.2	41.4
	AS3	X	S1796A-H2	61.3	56.5
	S1796A	X	AS3-H1	3.6	~0
	S1796A	X	AS3-H2	2.5	~0
Heat shock at 54°C	AS3	X	S1796A-T1	53.5	46.7
	S1796A	X	AS3-T1	35.8	33.7
	S1796A	X	AS3-T2	7.5	5.4

IV. DISCUSSION

A. Nutrient Requirements and Adaptation at Elevated Temperatures

It has previously been found that optimum growth of yeast is achieved by alteration of the media with different incubation temperatures. Stier and Scalf noted an increased lipid requirement for anaerobic growth of *Saccharomyces cerevisiae* at elevated temperatures.¹⁷⁷ Pine found that at 35°C the yeast *Histoplasma capsulatum* is much more sensitive to vitamin deletions, mainly thiamin, than at 25°C.¹³¹ The optimum-growth-rate temperature of yeast has been reported to be influenced by varying the concentration of ammonium chloride,^{55, 164, 22} potassium chloride,⁹ and sugar.¹⁵⁵

In the study by White and Munns yeast was observed to grow exponentially in synthetic medium over the temperature range of 20°C to 43°C.^{190, 191} However, in order to induce growth at 43°C, a larger inoculum was required. The marked differences between the above study and the investigation reported herein is undoubtedly due to different strains of *Saccharomyces cerevisiae*.

In this study marked dependence on the amount of yeast extract in the medium (Figs. 2-5) was noted for growth at 40°C. This dependence was mainly manifested by occurrence of an initial death phase in low concentrations of yeast extract, which was not observed at the optimum-growth-rate temperature of 30°C. The growth rate was also influenced by the concentration of yeast extract, so that the optimum concentration at 40°C was much greater (in the neighborhood of 8% YED) than at 30°C (about 2% YED).

The observation that under certain conditions oleic acid can completely eliminate the initial death phase is probably related to the change of lipid chemistry that occurs in yeast at elevated temperatures.³⁰ The mechanism of action, however, still remains obscure. It is of interest to note that oleic acid⁵ and other lipids^{4, 6, 19} are required for anaerobic growth. Nevertheless, the effect described in this investigation cannot be simply explained as a decrease in the amount of available oxygen, since there is little change in the solubility of oxygen between 30°C and 40°C.¹⁴¹ It should also be remembered that the petite variant can grow adequately without addition of oleic acid in low concentration of YED at 30°C (Fig. 1).

The death phase at 40°C was noted even in the absence of yeast extract, although little loss of viability was observed when yeast was incubated in buffer at this temperature in the absence of both yeast extract and dextrose (Fig. 13). This latter finding seems difficult to reconcile with the observations that increased sugar concentration provides a protective effect against lethal temperatures (Ref. 136, p 243; Ref. 157). The investigations by Schelhorn and others, however, were usually performed at above-maximum-growth temperature, in contradistinction to

this experiment. Since, as previously mentioned, autolysis is quite marked at around 40°C,⁷⁷ it seems justified to assume that the buffer was enriched with yeast extract from "leaking" cells, although none was furnished initially.

With the above facts in mind, this death phase is strikingly similar to cell death occurring by "unbalanced growth,"^{11, 178, 149} i.e., death occurring when certain biochemical mutants are incubated in a medium devoid of their specific growth requirements.

An initial decrease of viability has also been observed when cells are grown in the presence of toxic materials.^{70, 8, 21} To explain the onset of proliferation, after the initial death phase, two mechanisms are usually considered: (a) genotypic adaptation, i.e., selection of resistant mutants, and (b) noninheritable phenotypic adaptation, i.e., de nova alterations of existing cells.

Likewise, the increased ability of acclimatized yeast to perform biological functions at elevated temperatures has been previously reported by Zikes,²⁰⁷ Euler and Syanberg,⁵⁰ Staiger and Glaubitz,¹⁷⁵ Khlebnikova and Bolondz,⁸¹ Petite,¹³⁰ Chaturvedi,²⁸ and Guillot,⁶² and more recently by Loginova,^{99, 100} and Fang, Tang, Tsai and Wu.⁵² Christophersen and Precht have extensively studied the effect of temperature on the yeast Torulopsis kefir and, although no shift of the optimum temperature for growth of adapted strains was observed,³¹ changes of heat resistances and enzymatic properties occurred.^{135, 136}

In the study reported herein adaptation is indicated by the increase of viable cells in 1% YED after an initial death phase, and by the acquired ability of cells to grow in 0.5% YED after being acclimatized in 1% YED at 40°C. These results are more consistent with phenotypic adaptation, because if any thermophilic mutants were originally present in the inoculum, growth would eventually have proceeded in 0.5% YED. Also, the high degree of reversibility would indicate that these mutants would have a marked disadvantage at lower temperatures. It should be remarked, however, that selection has not been conclusively disproven.

One may conclude that growth at elevated temperatures results in an increased nutrient requirement which may be eliminated by induced adaptation.

B. Induction of Petites by Growth at Elevated Temperature

1. Population Analysis

In a population of cells the normal yeast cells are constantly mutating to the petite variant. The normal cells, however, enjoy a selective advantage, which, if large enough compared to the mutation rate, will result in a steady-state condition of a constant fraction of petites. This is what occurs normally at 30°C. However, if the mutation rate is sufficiently large, the petites will eventually outgrow the normal cells, as illustrated by growth at 38°C (Fig. 9). It is also possible that an absolute decrease in number of normal cells can result, as found with incubation at 40°C (Fig. 9). Therefore the resulting fraction of petites occurring by growth after a fixed time as a function of temperature, as delineated in Fig. 11, involves all the above three situations.

One may ask if the decrease of normal cells found at 40°C is due to the lethal effect of the temperature, or to mutation to the petite variant. This is satisfactorily answered by examining the results of the single-cell analyses (Table I), which indicate that the original normal cells are primarily lost by lethality and not by mutation.

Many similarities exist between the induction of petites by euflavine⁴² and by growth at temperature in the neighborhood of 40°C. In both cases no increase of petites occurs unless the cells are proliferating. Furthermore, there is a qualitative resemblance in that there is a greater probability that a bud will mutate than that the mother cell will. As indicated by the presence of "scalloped" or mixed colonies and from the results of single-cell analyses, both methods of induction produce an "unstable" state, i. e., a state in which a high mutation rate was preserved after the cells were removed from the inducing agents. A further possible parallel may be that the lethal and mutagenic properties are independent, as shown with euflavin by Marcovitch,^{112, 113} and by the experiment at 45°C in which lethality was observed with no increase in the percentage of petites. Nevertheless, there are a few significant differences which may be of theoretical importance. Before the details are discussed, a few remarks should be made about induction in general.

In a growing culture of yeast the number of normal cells, N_x , and petite cells, n_x , may be given as a function of the number of generations, x , by the differential equations

$$\frac{dN_x}{dx} = N_x \beta \ln 2, \quad (1)$$

$$\frac{dn_x}{dx} = n_x \lambda \ln 2 + (1 - \beta) N_x \ln 2, \quad (2)$$

where β is equal to the rate of increase of the log to the base 2 of the

number of normal cells. In other words, instead of the normal cells' doubling in number at each generation, a certain fraction mutates to the petite variant. The number of petite cells growing at a reduced rate λ can therefore be represented by Eq. (2). On the assumption that β and λ are constant, the solution for the number of petite cells as a function of the number of generations, x , is

$$n_x = N_0 2^{\lambda x} + N_0 \frac{1 - \beta}{\beta - \lambda} (2^{\beta x} - 2^{\lambda x}). \quad (3)$$

By taking the initial slope of the number of total cells as indicative of the generation time, one can plot the number of normal cells as a function of the number of generation. When this is done for X320 grown at 38°C (Fig. 9) it is found that β has two values--initially 0.82, and after about four generations 0.425 (Fig. 17). If these two values are used for β , and λ is allowed to equal 0.64, the number of petite cells determined theoretically by Eq. (3) agrees with the observed number (Fig. 17). One can therefore adequately describe the increase of petite cells in a growing culture at 38°C by assuming that the normal cells are constantly mutating to the petite variant and that the petite cells are growing at a reduced rate.

2. Mutation Rates

In order to quantitate the induction of petites, Marcovitch has defined the "mutation rate," π , as the probability that a bud taken at random from a normal cell will be a mutant.^{111, 112} This should not be confused with the classical definition of mutation rate, α (Luria and Delbruck,¹⁰²), which is usually given as the probability of mutation per unmutated cell per generation. It is of interest that the relationship of the two definitions is (Appendix I):

$$\pi = 2(1 - E^{-\alpha}).$$

The Marcovitch definition will be used throughout this paper.

The mutation rate of a steady-state culture with a constant fraction of petites, f_p , is given by Marcovitch¹¹² as

$$\pi = f_p(2 - 2^{\lambda}), \quad (4)$$

where λ is the selection rate, defined as the ratio of the growth rate of petites to the growth rate of the normals. In the derivation of the above formula it is assumed that once a petite cell is formed, it will grow at a constant reduced rate. This assumption, however, may be only approximate, since Harris has found that in certain media the petites are characterized by a low initial growth rate, which increases after considerable growth.⁶⁵ He also found a wide spectrum of generation times

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Fig. 17. Experimental points and the calculated curve of the number of petite cells as a function of the number of generations, x . Also shown is the experimental curve for the number of normal cells (see text).

with different petite isolates of the same culture. With these facts in mind, one sees that the mutation rate calculated by the above formula is only approximate, and probably lower than the true value. With $\lambda = 0.71$ (Fig. 1), the calculated values of the mutation rates of various diploid strains grown at 30°C in 6% YED are given in Table IV.

If the mutation rate is higher, so that the petites are outgrowing the normal cells, as at 38°C, a different method of calculation must be employed. By the use of the relationship given by Marcovitch,¹¹²

$$N_x = N_0 (2 - \pi)^x,$$

and Eq. (1), one can demonstrate

$$\pi = 2 - 2^\beta, \quad (5)$$

where β and its method of calculation are described above.

In Table IV the results of such calculations are shown for various strains grown at 38°C (see Fig. 9). For example, these calculations show that in a steady-state condition about 90% of the buds produced by normal cells of strain X495 are petite mutants. Implicit in these calculations are the assumptions that lethality and cluster formation are independent of the cell type.

As noted in Table IV and Fig. 11, the relative order of mutability of the various yeast strains is different at different temperatures. For example, strain X495 has the highest mutation rate for all the temperatures studied except 36°C (Fig. 11).

Table IV

Mutation rates at 30°C and 38°C for various diploid strains

Temperature	Strain		
	X320	X674	X495
30°C	0.004	0.01	0.037
38°C initial (4 to 7 hours)	0.14	0.07	0.15
38°C steady state (7 to 18 hours)	0.65	0.39	0.87

3. "Dilution" Model

As can be clearly seen in Table IV, the mutation rate at 38°C is initially very low, and after approximately four generations reaches a higher steady-state level. This change of mutation rate, which is usually not found with the induction by certain acridines,^{111, 112} can be best interpreted with various kinetic "dilution" models. Such models assume that there are initially a number of self-reproducing particles, which, under inducing conditions, cease to reproduce, or reproduce at a slower rate in relation to cell division. As a consequence, there is an increasing probability that a cell will possess either no particles or an insufficient number of particles as cell division proceeds and will therefore mutate. If there is a sufficient but low number of particles, the cell may produce both normal and mutant buds, and a resulting colony that is "scalloped." Such models have been applied to explain the elimination of kappa particles from *Paramecium*¹³⁸ and the reversion of "long-term adapted" yeast.^{174, 173, 172, 25, 24}

A general equation of this type, as modified from Spiegelman, DeLorenzo, and Campbell¹⁷³ can be given as follows: If it is assumed that a self-reproducing particle has a probability p of being received by a bud from the mother cell, and there are originally an average of θ_0 particles per cell growing at a reduced rate a , then the number N_x of normal cells at the X generation can be given by

$$N_x = N_0 \sum_{j=v}^{\infty} \sum_{i=0}^x {}^x C_i f_j(\theta_{x,i}),$$

where

v = minimum number of particles necessary for a cell to be normal,

$${}_x C_i = \frac{x!}{i!(x-i)!},$$

$$\theta_{x,i} = \theta_0 2^{ax} (1-p)^x \left[\frac{P}{1-p} \right]^i,$$

and $f_j(\theta_{x,i})$ is the probability of a cell's having exactly j particles if the mean is $\theta_{x,i}$. In order that the number of particles will not increase in the mother cells, i.e., the growth rate of the particles is not greater than the effect of dilution, the following condition must be met:

$$(1-p) < 2^{-a}.$$

As previously proposed by Spiegelman, the distribution function, $f_j(\theta_{x,i})$, can be assumed for the sake of simplicity to be equal to the Poisson distribution. The general formula can now be given as

$$N_x = N_0 \sum_{j=\nu}^{\infty} \sum_{i=0}^x x C_i \frac{(\theta_{x,i})^j}{j!} \exp(-\theta_{x,i}). \quad (6)$$

The results of the mass-culture experiment of X320 grown at 38°C (Figs. 9, 17) is used to illustrate the application of the above model to the induction of petites. However, this analysis is not presented in order to test the model, but rather--on the assumption that the model is correct--to determine the value of certain constants. The values of θ_0 and $\underline{a}$ can be accurately determined by using Eq. (6) and assuming a certain ν (Appendix II). The values of ν and p , however, do not appreciably affect the shape of the theoretical curves. Nevertheless, the best fit of Eq. (6) applied to the results of the X320 experiment leads to the values;

$$\nu = 1; \quad p = \frac{1}{2}, \quad \theta_0 = 3.36; \quad \underline{a} = 0.425.$$

This is shown in Fig. 18 along with the best-fit curves for $\nu = 1$, $p = 1/3$ ($\theta_0 = 3.36$, $\underline{a} = 0.425$) and $\nu = 2$, $p = \frac{1}{2}$ ($\theta_0 = 2.12$, $\underline{a} = 0.713$). Correspondingly, on the assumption of $\nu = 1$, the values of θ_0 for strains X674 and X495 (Fig. 9) are 2.04 and 3.54 respectively.

As can be seen in Fig. 18, the theoretical curves tend to approach the limiting slope in a smooth continuous fashion. However, the experimental results are best described by two straight lines (Figs. 9, 17). Whether or not this discrepancy disproves the model is still uncertain.

It should also be mentioned that these values of θ_0 are not derived from cells at time equal to zero, but when $x = 0$, i.e., when the cells begin to proliferate. Since there is a slight initial death phase (about 80% of the cells surviving), it is possible that the original number of particles per cell was altered.

Although the results of the single-cell experiment are as yet insufficient for a complete analysis, a more sensitive evaluation of certain constants can be obtained from these data than from the mass-culture technique.¹⁷³ Firstly, the approximate value of p may be calculated as follows: The average number of particles remaining in the x -generation buds of the daughter series can be given as

$$\theta_0 2^{ax} p^x.$$

On the other hand, the average number of particles in x generation of the sister series is given by

$$\theta_0 2^{ax} p(1-p)^{x-1}.$$

If the fraction of normal cells amongst x -generation buds of the daughter series is equal to the fraction of normal cells in the x' generation of the sister series, then it can be shown

$$2^{ax} p^x = 2^{ax'} p(1-p)^{x'-1}.$$

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Fig. 18. Theoretical curves of the "dilution" model, giving the number of normal cells as a function of the number of generations, x ; ν is the minimal number of particles required for the normal phenotype, and p is the probability that any given particle is transmitted to the daughter cell. Experimental points are for X320 at 38°C.

Although the data as shown in Table I are still of an approximate nature, one may take x equal to 2 for the fraction of normals to be on the order of $1/3$, and x' correspondingly may be in the neighborhood of 8 or 9. If the value of a is taken, as a first approximation, to be equal to the value calculated above for X320 at 38°C , then p can be shown to be equal to about $1/3$. This value does not seem unreasonable, since at these temperatures it has been shown that the mother cell retains more than half of its cytoplasm during division.²³

If one now assumes for simplicity that only one particle is necessary for a cell to be normal, the initial average number of particles can also be approximately calculated from the results of the single-cell analysis by using the previous equations. The results of such calculations lead to about four to six particles per cell.

Thus, on the basis of the above model, one is led to the conclusion that the number of self-reproducing particles per cell is low, around 2 to 6 per cell. This is in agreement with previous suggestions of various investigators (Ref. 26, p125 ; Ref. 39, p 33 ; Ref. 133). Slonimski has discussed other methods for the evaluation of the number of the self-reproducing particles.¹⁶⁸

It is quite possible that the above model is only an approximation of the real situation. Another model that could be considered is one in which the self-reproducing particles are attached to the cell wall, and the petite mutants develop from an area of the mother's cell wall which is devoid of such particles. Direct verification of the exact mechanism awaits a cytological investigation.

The induction of petites by growth at temperatures in the neighborhood of 38°C cannot be readily explained by the inactivation or inhibition of the cytochrome oxidase system, as this temperature lies far below the maximum for oxygen utilization.^{180, 31, 85} One must therefore conclude that the elevated temperatures act primarily by inhibiting enzyme formation. This is in correspondence with the results of various authors who have found that adaptive enzyme formation is more sensitive to elevated temperatures than enzyme activity present in adapted cells (see review by Knox,⁸³ Bernheim¹⁸). With this in mind it would be of interest to investigate the adaptation to oxygen of anaerobically grown yeast (see recent papers by Slonimski and de Robichon-Szulmajster,¹⁶⁹ Sels,¹⁶⁰ and Hebb and Slebodnik⁶⁷ at elevated temperatures, especially since this adaptation has been found to be blocked by euflavin.¹⁶⁸ Such investigations would help elucidate the site of action of elevated temperatures in the induction of petites, and specifically, if it is related to the meta-enzyme system, i. e., the catalytically active surface as proposed by Slonimski¹⁶⁸ and by Ephrussi⁴⁰ which may be responsible for the production of cytochrome oxidase.

C. Induction of Petites by Heat Shock

The production of petites by heat shock at 54°C differs from that in the supraoptimum-sublethal range in that growth is unnecessary in the former. A possible interpretation of this difference is that the division of self-producing particles is merely inhibited at the lower temperatures, while at the higher temperatures these particles are inactivated.

The decrease of oxygen consumption at temperatures in the vicinity of 50°C is evidence that the cytochrome oxidase system is inhibited or inactivated in this range.^{183, 32, 3} It has also been reported that if yeast is heated at 80°C for 20 minutes, the cytochrome a and b bands disappear, while the cytochrome c band remains.^{47, 117} This is of extreme interest if one remembers that the petite variant is lacking in cytochrome a and b, but retains the thermostable cytochrome c.

There, from a review of the literature, it is apparent that the cytochrome oxidase system is inactivated in the same temperature range in which petites are induced in nonproliferating cultures.

The heat induction of petites is probably a reflection of the thermolabile property of yeast mitochondria. This is interesting since it has long been known that the mitochondria of plants and animals are sensitive to elevated temperatures. Heat shock around 40° to 50°C has been reported to produce such changes as reduction of stainability, change of shape, and complete fragmentation and disappearance of mitochondria in hepatic and renal epithelium cells of frogs, (Policard,^{133a}, 1912), tissue-culture cells of chick embryos,^{92, 54} peas and pancreas cells,³⁵ iris, tulip, and the water mold *Saprolegnia*,^{134, 51} sea urchin eggs,³⁸ guinea pig pancreas,¹¹⁴ liver cells of fish,¹⁰⁵ rat thyroid,¹⁰ and insect larvae.⁷⁴ *In vitro* studies have also indicated the temperature-sensitive nature of mitochondria.^{34, 20} Jefferson tentatively concluded that the heat injury is due primarily to the breakup of mitochondria.⁷⁴ It should be pointed out, however, that the petite mutants may not have completely lost their mitochondria, as indicated by the existence of nonstainable refractile granules, and the presence of certain sedimentable enzymes,⁹⁸ but the mitochondria may merely be functionally altered. It is also possible that the mitochondrial population is heterogeneous and the mutation is the result of the loss of a fraction carrying cytochrome oxidase.⁴⁸

It seems appropriate to consider possible quantitative models for the analysis of the induction of petites at lethal temperatures. A simple model can be given as follows: Initially there is an average of θ_0 particles per normal cell, and these are inactivated by a first-order reaction, i. e., the same fraction of all particles that are surviving at any given time will be inactivated in the next time unit. Let it also be assumed that a cell has "mutated" when there is an insufficient number of particles per cell. If the inactivation of the particles is independent of cell death, and the sensitivities to heat of both normal and petite cells are of the same order of magnitude, then the fraction of petites will be very low for short exposures, increasing

concavely upwards with time of exposure to inducing agent. The induction of petites by ultraviolet irradiation is approximated by this model.^{147,132,133,154} Although the data (see Fig. 15) are still capricious, it can be seen that the above analysis does not seem to apply to the induction of petites by lethal temperatures, but is best approximated by a linear relationship, i.e., $\theta_0 = 1$. It would be presumptuous to perform an extensive analysis of the existing results, and a more complete interpretation awaits further data.

D. Other Examples of Temperature-Induced Non-Mendelian Variants

Lederberg has proposed the generic term plasmids for "extra-chromosomal, intracellular hereditary factors, irrespective of their further identification as plasmagones, viruses, self-reproductive organelles, endosymbionts, etc."^{88,89} As pointed out in his review, the disinfection of plasmids by differential heat treatment is quite prevalent. The mechanism may involve either in vivo heat inactivation or elimination by dilution if elevated temperatures inhibit or retard growth of the plasmids in respect to cell division. Since the heat induction of petites may be looked upon as a typical example of this phenomenon, it is appropriate to consider similar situations in other systems.

First, it is well documented that certain plant viruses may be eliminated by exposure of diseased plants to temperatures 10° or 20°C above normal, and more than 30 different types of virus-infected plants have been reported to be cured by this method (see review by Kassanis,⁷⁹ Thomson,¹⁷⁹ Benda and Naylor,¹⁷ However, some cures are temporary and symptoms may reappear when the plants are returned to normal conditions, indicating an incomplete disinfection of the virus. Such treatment may also be associated with heat injury to the host, resulting in an increase in the number and size of lesions, and other varied effects (see e.g., Kassanis,^{79,80,158,205}

Similarly, as in plants, viruses in insects may be either permanently or temporarily eliminated by elevated temperatures.^{86,110} L'Heritier and co-workers have also found that the nonpathogenic virus causing CO_2 sensitivity in Drosophila is temperature-sensitive.⁹³ Rasmuson has reported negative results with temperature treatment of Drosophila melanogaster, which have cytoplasmic factors causing increased sensitivity to anaesthetics.¹⁴⁵ Because of this, and the inability to produce infection by transplantations, he has concluded that it is unlikely that this phenomenon is due to a virus or viruslike particle. In Drosophila bifasciata, Magni has reported that high temperature inhibits or partially inactivates cytoplasmic units that control the production of male offspring.¹⁰⁶ In other species of Drosophila, however, temperature has no effect on the maternally inherited "sex ratio."¹⁰⁹

By incubation at supraoptimum temperatures viruses may also be inhibited in growing cells of human tissue culture (poliomyelitis¹⁰³) and bacteria (staphylococci-phage⁸⁴ coli-phage¹⁰¹).

In Paramecium aurelia there exist killer clones that contain various plasmids (κ , μ) which result in the production of toxic effects on sensitive strains (see general reviews by Beale¹³ and Preer¹³⁹). These plasmids may be eliminated from certain killer animals by a number of methods, including rapid growth, growth at elevated temperatures, and heat shock to nondividing cultures.^{170, 171, 137, 138, 90, 165, 14} It is interesting that the related, but not identical, paramecin particles are also extremely temperature-labile, as determined by in vitro studies.^{185, 36}

With several pure lines of tomatoes, a high temperature during germination increases the frequency of a non-Mendelian variant, "rogue," which is due to a transformation or reorientation in the seed.⁹¹ By short heat treatments an intermediate type, or half-rogue, may be induced, which may be "evidence that different equilibria between the competing plasmagenes can be attained in the cells." It is also possible to interpret this result as the existence of two distinct types of plasmagenes in the "normal" plant, one more thermolabile than the other. Short heat treatment may inactivate only one type, resulting in the stable intermediate half-rogue.

Pringsheim and Pringsheim were able to produce permanent apoplastidic races in certain strains of Euglena gracilis by growth just below the maximum temperatures for multiplication.¹⁴⁰ Short periods of heat shock were not effective. Unfortunately, detailed genetic investigations in Euglena are not available, and the site of the mutation is unknown, but it is in all likelihood similar to the previously described loss of plasmids.

In the fungi Schizophyllum¹²⁸ and Aspergillus¹⁰⁷ incubation at higher temperatures causes an increase in the mutation rate to certain non-Mendelian variants. Whether or not these mutations are due to a loss of plasmids is still uncertain, although such interpretation does not seem objectionable.

It should be pointed out that negative results with heat treatment do not eliminate the possibility of the existence of plasmids, since the host may be more sensitive than the plasmids to elevated temperatures. Therefore, such negative results as were reported with non-Mendelian variants of Chlamydomonas¹⁵² and Drosophila^{145, 109} do not exclude the plasmid hypothesis.

Also, the occurrence of mutations after heat treatment does not always imply the loss of plasmids; consequently, independent methods should be employed to determine non-Mendelian characteristics. Many unstable alleles are known which are affected by temperature (see, e.g., Sand¹⁵³ Ishikawa,⁷² Peterson,¹²⁹ There are a multitude of reversible changes that occur with variation of temperature, some being controlled by single temperatures-sensitive alleles (see reviews by Precht, Christophersen, and Hensel,¹³⁶ Wagner and Mitchell.¹⁸⁴ Furthermore, there are many reversible changes that are controlled, at least in part,

by cytoplasmic factors and are strongly influenced by temperature, such as mating types, antigens, and dauermodifications in Paramecium. As pointed out by several authors, these determinants cannot easily be accounted for in terms of plasmids, as for killer characters. (See Beale, ^{13, 15} Preer, ¹³⁹)

With the use of temperature shock and other agents on the larvae of Drosophila many changes occur which mimic known Mendelian mutants, but are not transmitted to the sexual offspring. Whether or not these "phenocopies," as termed by Goldschmidt, ^{59, 60} are due to a loss of plasmids during the course of development of the insects is unknown, but such speculation is not inconsistent with some theories of somatic differentiation (see, e.g., Brachet, ²⁰ Ephrussi, ³⁹ Shaver, ¹⁶¹)

E. Heat Inactivation of Yeast

The heat-inactivation experiments of this investigation were directed primarily to the study of petite production. However, a few remarks should be made on lethality per se. The surviving fractions of various strains of yeast as a function of time of exposure to lethal temperatures have been previously reported by Rahn and Barnes, ¹⁴⁴ Beamer and Tanner, ¹⁶ White, ¹⁹⁰ Weinfurtner and Voerkelius, ¹⁸⁶ and Wood. ¹⁹⁷ In many cases it was found that a small fraction of the population was much more resistant. This may have been the result of clustering of cells, or sporulation. As indicated by this investigation, the variations of sensitivity and shapes of survival curves are probably due not only to the different strains employed, but also to variation of the previous growth conditions. The prodigious dependence of heat sensitivity on the age of the yeast culture has been reported as early as 1895, ¹⁵⁹ and more recently discussed by Christophersen and Precht ^{31, 33} and Rosenberg and Wood. ¹⁵¹ Thus, by this investigation and the review of the literature it appears as if the heat inactivation of yeast is highly complex, and strongly dependent on the previous history of the cell.

Many different mechanisms have been proposed to explain the lethal effects of elevated temperatures (see recent reviews by Precht, Christophersen, and Hensel, ¹³⁶ and Wood ¹⁹⁷). Although it would be naive to assume that heat inactivation is due entirely to the destruction of a particular thermolabile site in the cell, it is of interest to consider whether cell death is due primarily to damage of the cytoplasm (i.e., enzyme or protein inactivation) or to the genetic mechanism. By the examination of the survival curves of microorganisms, Rahn concluded that thermal inactivation is the result of destruction of a small number of redundant "molecules," and subsequently suggested that the genetic apparatus is the site in question. ^{142, 143} However, in a recent review, Christophersen (Ref. 136, p253) pointed out various difficulties of Rahn's hypothesis and reviewed many papers which indicate that cell death is due to inactivation of the cellular enzymes.

By the use of additivity studies in which two different lethal agents are applied to a living system, it is possible to determine whether each agent acts at least partially on the same site. Lethality studies on yeast with heat plus ultraviolet,³ heat plus x-ray,^{196, 197} and uv plus x-ray^{182, 37} indicate that there is some interaction and overlap in sites sensitive to the three agents. By assuming that all damage of x-ray inactivation is nuclear, Wood has concluded that the lethal effect of elevated temperatures is partially also nuclear.¹⁹⁷

The results of this investigation throw little light on this problem. However, if one assumes that thermal inactivation is completely due to damage of the genetic mechanism, independent of the cytoplasm, then the normal and petite strains should exhibit the same response to lethal temperatures. But this is not the case; therefore, we must conclude that the damage resides at least partially in the cytoplasm or is modified by it.

The quantitative results of the heat inactivation of the normal and petite strains, as illustrated in Fig. 16, suggest the following: If one assumes that a certain number of thermolabile sites have to be inactivated in order for death to occur, and that the petite has a relatively smaller number or an altered form of sites, then a petite culture's response may be similar to that of a population of normal cells that have survived a period of heat exposure. Also, since there is a difference between the responses of the strains X320-S2-1 and X320-S2-15, it is suggested that these thermolabile sites are lost or altered as vegetative growth of the newly formed petites proceeds.

From the above results it seems plausible to identify these thermolabile sites with the mitochondria. Although this seems highly speculative, it should be remembered that Jefferson has previously suggested this connection to explain his results with insect larvae, and to link the "enzyme" and "lipoid liberation" theories of heat injury.⁷⁴ Claude has shown that incubation of extracts of dried yeast at 40°C for 3 hours results in a different type of sedimentable particle.³⁴ There is also much evidence that the thermolabile property of ribonucleic acid⁵⁸ is responsible for initial effects of elevated temperatures.^{34, 20, 187, 18}

There is some disagreement on the exact nature of the mitochondria in yeast,^{66, 78, 121} but they are believed to be low in number,^{122, 12, 64} and to undergo variation with the age of the culture.^{49, 76, 78, 66} The above may help to explain the low multiplicity of the survival curves, and the large variations in sensitivity.

This highly speculative theory can be summed up as follows: If the mitochondria are partially destroyed by lethal temperatures, a mutation to the petite variant occurs; whereas, if the mitochondria are completely destroyed, lethality results.

Differential responses of normal and petite strains occur with various physical and chemical agents. Petite strains have been reported

to be more sensitive to x-ray inactivation by Gonzales and Barron,⁶¹ Moustacchi,¹²⁰ and O'Brien,¹²⁵ although no significant difference was observed by Raut and Simpson¹⁴⁸ and Wood and Taylor.¹⁹⁸ Pittman has observed an increased sensitivity of petites to ultraviolet irradiation.¹³² Raut observed no significant difference in uv response,¹⁴⁷ and Elkind and Sutton's survival curves³⁷ seem to indicate that the petite strains they employed were more resistant than the normal strain. The petite variant is more sensitive to the respiratory inhibitors, sodium azide, dinitrophenol, p-nitrophenol,^{203, 204} potassium cyanide, and malonic acid¹⁶³ in that their growth is inhibited in concentrations which do not appreciably affect the normal strain.

V. SUMMARY AND CONCLUSIONS

1. The growth of yeast at 30°C, the optimum-growth-rate temperature, is relatively independent of the percentage of yeast extract. However, at 40°C no growth was observed in 0.5% YED (0.5% yeast extract, 4% dextrose), unless the inoculum consisted of cells which were previously grown at 40°C. In 1% YED, growth proceeded only after an initial death phase, which could be eliminated by the addition of 10⁻⁴ mole/ml of oleic acid. In higher percentages of YED, growth is relatively rapid.

No death phase was observed at 40°C if the yeast were incubated in buffer that prevented proliferation. Thus it would appear that this cell death is similar to lethality occurring by "unbalanced" growth.

The conclusion based on these observations is that growth at elevated temperatures results in an increased nutrient requirement which may be eliminated by induced adaptation.

2. When yeast is grown at supraoptimum temperatures, there is a great increase in the fraction of respiratory-deficient mutants (petites). However, in the absence of proliferation no increase was observed. By the analyses of the growth curves of the mass cultures and the single-cell experiments, it was shown that this increase was not due to selection, but to induction by the elevated temperature. This high mutation rate continued for many generations, even after the cells were returned to the optimum temperature (30°C).

3. When the temperature of exposure was raised to above-maximum-growth temperature (45°C) no induction was observed. However, if the temperature was still further raised to extremely lethal temperatures (54°C), it was found that many petite colonies arose among the survivors. The conclusion that lethal temperatures induce petites is based on the observations that isolated petite strains were more sensitive to the lethal effect of elevated temperatures, and that an absolute increase in the number of petite colonies occurred after a short heat exposure.

4. Crosses of petite strains with each other and with normal strains were undertaken in order to determine the nature of the petite variants. It was concluded from the results of the genetic analyses that the petites obtained by heat shock and growth at elevated temperatures were similar, if not identical, to the vegetative petites occurring spontaneously or induced by acriflavine.

5. The results of the induction experiments can be interpreted as follows: There is initially in the normal cell a number of self-reproducing particles, which under supraoptimum temperatures cease to reproduce, or reproduce at a slower rate in relation to cell division. These particles can also be lost at lethal temperatures by heat inactivation. Therefore, a cell which possesses either none or an insufficient number of these particles mutates to the petite variant. On the assumption of the validity of a quantitative model (modified from Spiegelman et al.), it was found that the number of self-reproducing particles per cell is low, approximately 2 to 6 per cell.

6. In a review of the literature it was noted that many other examples of temperature-induced non-Mendelian variants occur in both unicellular and multicellular organisms.

7. The sensitivity of yeast to heat inactivation was found to be strongly influenced by their previous growth conditions. It is suggested that the mitochondria play an important role in the temperature inactivation of yeast.

ACKNOWLEDGMENT

The author wishes to express his sincere appreciation to Dr. Robert K. Mortimer for his encouragement, criticisms, and constructive suggestions offered during the course of this investigation. Thanks are also extended to Dr. Cornelius A. Tobias for his guidance, and to Mr. Morton D. Davis and Miss Kwan Hsu for helpful discussions on mathematical phases of this work.

Appreciation is also due to the directors of Donner Laboratory and to the Atomic Energy Commission for making available laboratory space and equipment.

This work was supported in part by the U.S. Atomic Energy Commission.

During the course of this investigation the author was a Public Health Service Research Fellow of the National Cancer Institute.

APPENDIX I

The number dm of mutational events that occur in a growing culture during an interval of the division cycle dx is given¹⁰² as

$$dm = a N_x dx ,$$

where N_x is equal to the number of unmutated cells, and a is the probability of mutation per unmutated cell per generation. One can therefore express the rate of increase of the normal cells as

$$\frac{dN_x}{dx} = N_x \ln 2 - N_x a ,$$

which reduces to

$$N_x = N_0 2^x e^{-ax} .$$

Substituting¹¹² into the equation,

$$N_x = N_0 (2 - \pi)^x ,$$

one can show,

$$\pi = 2(1 - e^{-a}) .$$

When the mutation rate is small, the approximate relation is

$$\pi = 2a .$$

It should be noted that the number of mutational events that occur during one generation of N_x cells,

$$a \int_x^{x+1} N_y dy ,$$

is not equal to the number of mutants formed,

$$\pi N_x .$$

APPENDIX II

In order to illustrate the method of determining the constants of Eq. (6) with the experimental results, a special case in which ν is equal to unity is considered. Under this condition the equation reduces to

$$N_x = N_0 \left\{ 2^x - \sum_{i=0}^x x C_i \exp(-\theta_{x,i}) \right\},$$

in which the symbols have the same meaning as in Eq. (6). When x is large, $\theta_{x,i}$ is small and the above equation can be approximated by

$$N_0 \left\{ 2^x - \sum_{i=0}^x x C_i \left[1 - \theta_0 2^{ax} (1-p)^x \left(\frac{p}{1-p} \right)^i \right] \right\}.$$

Remembering that we have,

$$\sum_{i=0}^x x C_i = 2^x, \quad \text{and} \quad \sum_{i=0}^x x C_i (1-p)^{x-1} p^x = 1$$

one can simplify the above equation to

$$N_0 \theta_0 2^{ax},$$

and $\underline{a}$ is equal to β of Eq. (1) when x is large.

By plotting the experimental results as log to the base 2 of the number of normal cells as a function of the number of generations, one finds that the limiting slope is equal to $\underline{a}$. If a straight line is extended from this limiting slope to $x = 0$, the extrapolated value is equal to $\log_2 N_0 \theta_0$. With this method θ_0 and $\underline{a}$ can be accurately determined.

Although slightly more complicated, similar methods can be employed for ν greater than unity.

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