ON THE ORIGIN OF MITOCHONDRIAL MUTANTS: EVIDENCE FOR INTRACELLULAR SELECTION OF MITOCHONDRIA IN THE ORIGIN OF ANTIBIOTIC-RESISTANT CELLS IN YEAST

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ABSTRACT

In wild-type *Saccharomyces cerevisiae*, erythromycin and certain other antibacterial antibiotics inhibit the formation of respiratory enzymes in mitochondria by inhibiting translation on mitochondrial ribosomes. This paper is concerned with the origin of mutant cells, resistant to erythromycin by virtue of having a homogeneous population of mutant mitochondrial DNA molecules. Such mutant cells are obtained by plating wild-type (sensitive) cells on a nonfermentable substrate plus the antibiotic. Colonies of mutant cells appear first about four days after the time of appearance of established mutant cells; new colonies continue to appear, often at a constant rate, for many days. Application of the NEWCOMBE respreading experiment demonstrates that most or all of the mutant cells which form the resistant colonies on selective medium arise only *after* exposure of the population to erythromycin. It is suggested that this result is most probably due to intracellular selection for mitochondrial genomes. Resistant mitochondria arising from spontaneous mutation are postulated to be at a selective disadvantage in the absence of erythromycin; reproducing more slowly than wild-type sensitive mitochondria, they cannot easily accumulate in sufficient numbers in a cell to render it resistant as a whole. In the presence of erythromycin, resistant mitochondria can continue to reproduce while sensitive mitochondria cannot, until there is a sufficient number to make the cell resistant, i.e. to permit normal cell growth. The same phenomenon is seen with respect to chloramphenicol resistance. Intracellular selection is considered more likely than direct induction of mutation by the antibiotic, since mutant cells do not accumulate in the presence of erythromycin if the mitochondrial genome is rendered nonessential by growth on glucose or nontranslatable by chloramphenicol. Intracellular selection provides a mechanism for direct adaptation at the cell level, compatible with currently acceptable ideas of spontaneous mutation and selection at the organelle level.

A crucial step in the development of microbial genetics was the demonstration that most hereditary variants in bacteria arise by selection of spontaneous mutants rather than by adaptation induced by the selective agent. Of the several experimental designs used to distinguish between these alternatives, the respread-

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ing experiment of NEWCOMBE (1949) is especially elegant in design and simple in execution. Cells of *E. coli* sensitive to phage T1 were allowed to grow for a number of generations on plates in the absence of phage. The bacteria on some plates were then redistributed over the plate by spreading with a drop of saline, and all plates were sprayed with phage and counts made of the number of colonies of resistant mutants developing after further incubation. Colony counts were always higher on the respread plates, indicating that the resistant cells arose by spontaneous mutation during the prior growth in the absence of phage, forming clones of mutant cells. On the undisturbed plates, each mutant *clone* formed a colony, while on the respread plates, each mutant *cell* formed a colony.

If, on the other hand, the phage had induced the appearance of phage-resistant bacteria, then all bacteria present after the initial growth period would have been sensitive; respreading would have merely redistributed the members of a homogeneous population and the number of mutant colonies would have been the same on the spread and unspread plates. In this paper I show that this heterodox result is obtained for the origin of yeast cells with mitochondrially-inherited drug resistance, and is explicable in terms of orthodox spontaneous mutation of organelles followed by *intra*cellular selection.

Most laboratory strains of bakers' yeast (*Saccharomyces cerevisiae*) fail to reproduce and form visible colonies when plated on nutrient agar containing certain antibacterial antibiotics such as erythromycin or chloraphemicol and a nonfermentable carbon source such as glycerol, while colonies are formed on fermentable carbon sources such as glucose at the same drug concentration (WILKIE, SAUNDERS and LINNANE 1967). The antibiotics are believed to inhibit translation specifically on mitochondrial ribosomes, so that sensitive mitochondria produce a defective electron transport system and do not carry out oxidative phosphorylation (LINNANE, LAMB *et al.* 1968; WILKIE 1970; GRIVELL, REIJNDERS and DEVRIES 1971; MAHLER and PERLMAN 1971). Resistant colonies may be isolated from glycerol-drug plates, either as spontaneous mutants or after treatment of sensitive cells with mutagens. The mutants of interest for this paper are mitochondrial; their transmission genetics is distinctive (THOMAS and WILKIE 1968; LINNANE, SAUNDERS *et al.* 1968; COEN *et al.* 1970; BUNN *et al.* 1970), showing vegetative segregation in diploid heteroplasmons and loss of ability to transmit resistance or sensitivity when the cells are converted to cytoplasmic petite (ρ^-) mutants. The ρ^- mutation is known to involve the loss of part or all of the mitochondrial DNA information (MEHROTRA and MAHLER 1968; NAGLEY and LINNANE 1970), so that these experiments indicate that the antibiotic resistance mutation is an alteration of mitochondrial DNA. Similar mitochondrial mutants have also been selected in *Paramecium aurelia* (BEALE 1969; ADOUTTE and BEISSON 1970; BEALE, KNOWLES and TAIT 1972).

When established antibiotic-resistant yeast cells are plated on selective medium, visible colonies appear within two or three days. In contrast, when sensitive cells are plated on the same medium, spontaneous mutant colonies appear only after a lag of several more days (COEN *et al.* 1970). A similar lag is observed in *Paramecium aurelia* (BEALE 1969; ADOUTTE and BEISSON 1970).

This suggested that wild-type populations of cells might contain only very low frequencies of preexisting mutant cells, and that the spontaneous mutant cells which eventually appear arise primarily or exclusively *after* exposure to the drug. This has been verified by use of the NEWCOMBE experiment and evidence has been obtained that the antibiotic selects for the replication of drug-resistant mitochondria within the population of mitochondria in single cells rather than by inducing mutation to drug resistance. This hypothesis also explains the ease of selecting for resistant cells, which is unexpected since spontaneous mutation at ordinary rates should affect only one mitochondrion out of many in a yeast or paramecium cell.

MATERIALS AND METHODS

Strains: Saccharomyces cerevisiae, clones D6–1–2 *α arg met* (hereafter, D6 E^s) and D22–1–2 *a ade-2* (D22 E^s) are subclones of stocks supplied by DR. DAVID WILKIE. D6 E^r_{16} and D22 E^r_2 are spontaneous erythromycin-resistant mutants tolerating at least 5 mg erythromycin/ml YEPG; the parental E^s strains grow slowly on 0.01 mg erythromycin/ml and are completely inhibited by 0.1 mg/ml. Clone 41 *α his ura* (41 C^s) and its mutant derivative 41 C^r_{30} were supplied by DR. DAVID THOMAS; they tolerate 0.01 mg and >3 mg chloramphenicol/ml, respectively.

Media and Materials: Incubation was at 30°C, usually in 1% Difco yeast extract plus 2% Difco Bacto-peptone with 2% dextrose (YEPD) or 4% glycerol (YEPG) as carbon sources. In some experiments, YEPD and YEPG were made up in 0.05 *M* Sorenson's Na-K phosphate buffer at pH 6.2. The final pH of solidified YEPG was about 6.7 whether buffer was used or not; antibiotics did not change the pH. Minimal medium for prototroph selection of zygotes was essentially that of WICKERHAM (1946), with 2% dextrose (MMD). Solidified media were made with 2% agar. Erythromycin lactobionate was used as the purified antibiotic (kindly supplied by DR. ROBERT SINGISER, Abbott Laboratories, potency 670 μg erythromycin base/mg) or as Erythrocin IV (Abbott Laboratories, potency 573 μg base/mg plus 0.9% benzyl alcohol). Chloramphenicol (potency 1012 μg/mg) was purchased from Mann Research Laboratories. Antibiotics were added to molten medium at 45–55°C either as a powder or in aqueous or alcoholic solution to a final concentration expressed in mg base/ml medium. Experiments with membrane filters used either Sartorius Membranfilters (0.8 micron mean pore size) or Millipore RA filters (1.2 micron mean pore size).

Assay of cell numbers: Cell concentrations were determined by plating aliquots of appropriate dilutions of the cell suspension on YEPG or YEPD, and/or by direct counts in a hemacytometer or Petroff-Hausser chamber. In order to determine the number of cells on a plate or a filter, the surface of the plate or filter was washed repeatedly by adding a few ml of sterile water and rubbing with a glass rod; the pooled wash fluids were made up to 10 ml for counting. In most cases, three filters or plates selected at random were washed, and 1-ml aliquots of the wash fluid from each plate were pooled for counting.

Genetic analysis; A number of E^r mutants of stock D6, obtained in a NEWCOMBE experiment, were analyzed to determine if they were nuclear or mitochondrial. Each mutant was picked from a filter on YEPG plus erythromycin and grown up on YEPD. Mutants were then replica-plated onto YEPG plus 0, 0.1, and 1 mg erythromycin/ml to verify their resistance and onto MMD previously covered with a lawn of cells of stock D22 E^s for mating and prototroph selection of diploids. Random diploids from each mutant were taken from the latter plates, suspended in water, and streaked on MMD plates to obtain subclones. These subclones were replica-plated onto YEPG plus 0 and 1 mg erythromycin/ml to determine resistance *vs.* sensitivity for each subclone. Alternatively, random diploids were streaked onto YEPD plus 0.1 mg erythromycin/ml. The resulting colonies were covered with TTC agar (OGUR, ST. JOHN and NAGAI 1957); E^r colonies stain red, while E^s colonies stain pale pink. With either technique, the appearance of

both E^r and E^s diploids among the diploid progeny of a cross indicated vegetative segregation and probable cytoplasmic inheritance of the E^r mutation. Mutants showing vegetative segregation were then exposed overnight to 10 μg ethidium bromide/ml to induce cytoplasmic petite (ρ-) mutants, then streaked on YEPD. Petite mutants were identified by the small size and white color of their colonies. For each E^r mutant, one ρ-colony was mated to D22 E^r and analyzed for vegetative segregation as above; a mutant from which a ρ-derivative could be obtained which did *not* transmit E^r to its progeny was classified as a mitochondrial mutant. Tetrad analyses were performed as described by MORTIMER and HAWTHORNE (1969).

RESULTS

Kinetics of appearance of colonies of E^s and established E^r cells: The interpretation of the experiments described here depends in part on the kinetics of appearance of visible colonies on a plate during incubation. Colonies are scored as visible when they can just be distinguished with the naked eye or at low (less than 2×) magnification; they are usually at least 0.5 mm in diameter. When E^s and established E^r cells are plated at low densities on Millipore filters on YEPG, the first colonies are seen after 1½ to 2 days, and most are visible by 3 days.

As controls for the study of the appearance of spontaneous mutant cells in populations of E^s cells plated on YEPG plus erythromycin, a few established E^r

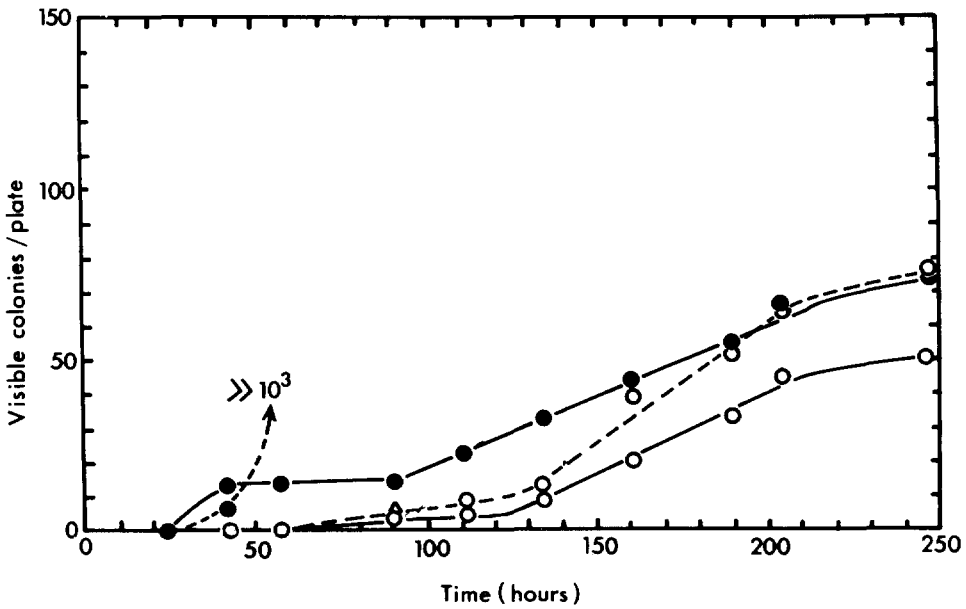

FIGURE 1.—NEWCOMBE experiment. About 10^5 cells of D6 E^s, or 10^5 D6 E^s plus 15 D6 E^r (●), were plated on Millipore filters on YEPG plates. After 25 hours (about 9 generations), the filters had approximately 5×10^7 E^r, or 6×10^7 E^s plus 10^3 E^r cells (the E^r cells were in about 15 clones). The filters were transferred to YEPG plus 1 mg erythromycin/ml; some were respread to redistribute E^r cells from their clones (- - -) and some were not (——). From this time (zero hours) on, visible colonies were counted daily. Counts are means of three filters. The E^s cells underwent about 3.5 residual generations after exposure to erythromycin.

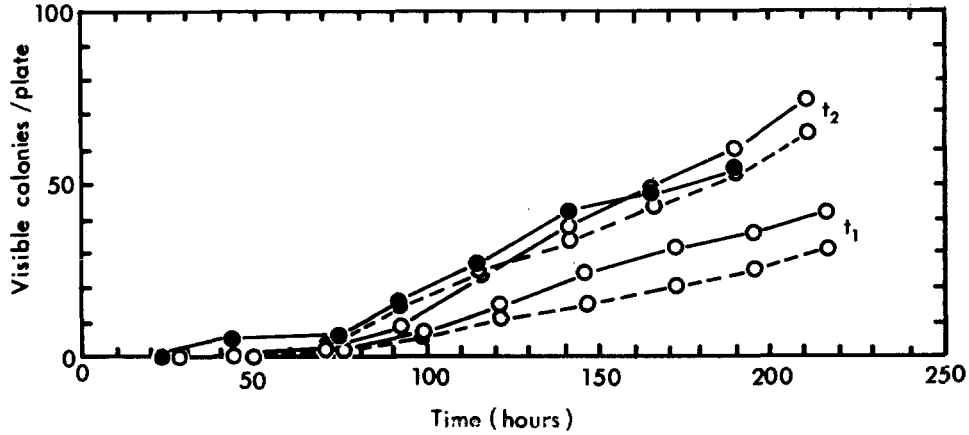

FIGURE 2.—NEWCOMBE experiment. About 3×10^5 cells of D22 E^s alone (○) or with about 5 E^r cells (●) were plated on Sartorius filters on YEPG. After 12 hours (t_1, just over 3 generations) some filters were transferred to YEPG plus 1 mg erythromycin/ml and respread (– – –) or not (——). Other filters were transferred to selective medium after 18 hours (t_2, 7–8 generations). Only the experimental filters are shown for t_1. Experimental data are means of three filters; the t_2 control data are from one filter.

mutant cells were plated together with E^s cells. The kinetics of appearance of colonies of these established E^r mutants can be seen in Figures 1, 2, and 4. The curves are of essentially the same form as those for E^r cells plated alone, except

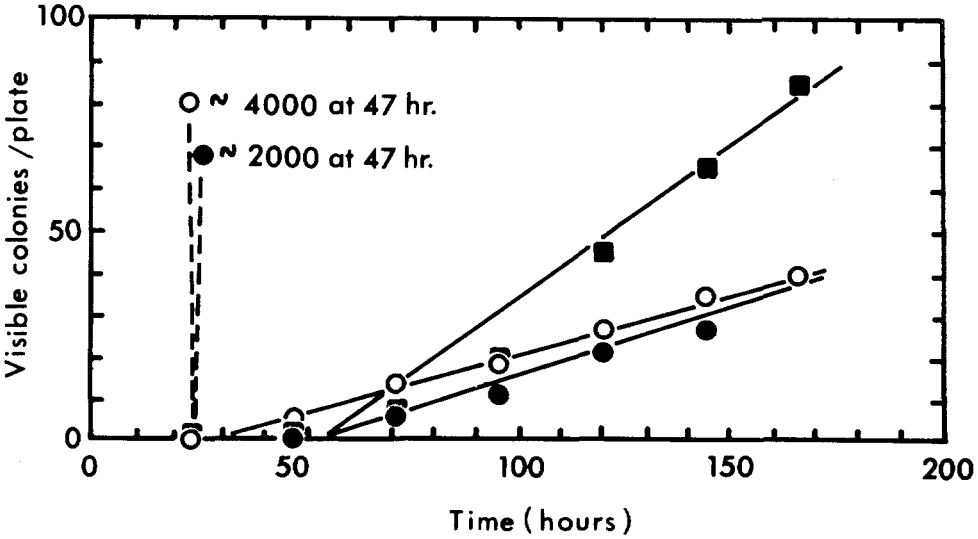

FIGURE 3.—Test for induction of E^r mutations by erythromycin in glucose-grown cells. About 8×10^4 cells of D6 E^s alone (——) or with about 24 cells of D6 E^r (– – –) were spread on Millipore filters and grown for 42 hours on YEPD (open symbols; about 14–15 generations) or on YEPD plus 1 mg erythromycin/ml (solid symbols; about 17–18 generations). At time zero, the cells were washed off the filters and aliquots containing about 1×10^7 E^s ± 2000–4000 E^r cells were plated on filters on YEPG plus 0.5 (circles) or 0.9 (squares) mg erythromycin/ml.

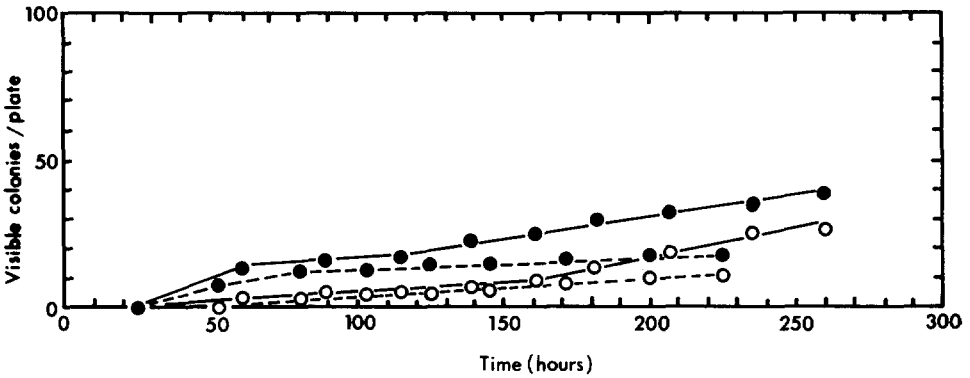

FIGURE 4.—Test for induction of E^r mutations by erythromycin plus chloramphenicol. D6 E^s cells, alone (○) or with a minority of D6 E^r cells (●) were grown in YEPG to log phase. Aliquots were then grown for an additional 6 hours in YEPG (about 2 generations) (——) or for 42 hours in YEPG plus 1 mg erythromycin and 3 mg chloramphenicol/ml (about 3 generations) (– – –). Aliquots containing approximately 10^8 E^s cells or 10^8 E^s plus 20 E^r cells were then (zero time) plated on YEPG plus 1 mg erythromycin/ml and visible colonies counted daily. Not shown are similar data for cells grown 6 hours or 18 hours in YEPG plus erythromycin and chloramphenicol.

that colonies often become visible from ½ to 1 day later. This is due in part to difficulties in distinguishing growing colonies against a dense background of non-growing cells; it may also reflect a small degree of inhibition of growth by the excess E^s cells (too small to be detected in controls described below). The curves rise to a plateau and then rise again one or more days later; the second rise is due to the appearance of spontaneous mutant E^r colonies, as is discussed next.

Kinetics of appearance of colonies from spontaneous E^r mutant cells: When filters bearing about 10^6 to 10^8 E^s cells are placed on YEPG plates containing 0.1 to 5 (usually 1) mg erythromycin/ml, either with or without several generations of prior growth on YEPG without erythromycin, the E^s cells undergo two to four, rarely six, generations of residual growth and budding in the first day or two, and then stop. The results of typical experiments are shown in Figure 1 through 4 (E^s cells, filters not respread). Colonies of new E^r mutants are first visible after two to four days; thereafter, new colonies appear each day for an indefinite period of time.

The first striking feature of these curves is the lag between the appearance of colonies of established E^r mutants on control plates (E^s plus E^r cells) and the appearance of new, spontaneous mutant colonies on the experimental plates or on the same control plates (second rise from the plateau). This lag is highly variable between experiments, ranging from one to four days, but is a consistent feature of all experiments. In my experiments, the lag is of much shorter duration than reported by COEN *et al.* (1970).

Following the lag, new E^r colonies appear with kinetics which are usually linear initially and for up to 250 hours. It is possible to calculate the rate at which E^r mutant cells arise, providing one assumes that each colony is produced by a

single E^r cell which begins growth and reproduction soon after being formed. Such rates, calculated from linear portions of the curves after correction for residual growth, range from 2.0 to 4.3×10^{-8} events $cell^{-1}day^{-1}$.

Mitochondrial basis of the spontaneous E^r mutants: The great majority of the colonies seen in these experiments consist of stable mitochondrial E^r mutants. A total of 114 different D6 mutants from one experiment were tested; the colonies had appeared at various times on YEPG plus erythromycin, ranging from 58 to 205 hours. Of 50 mutants selected from plates containing 0.1 mg erythromycin/ml, all were stably resistant; when crossed to D22 E^s tester, 46 gave a mixture of E^s and E^r progeny, indicating cytoplasmic inheritance, while 4 gave only E^s progeny, which could indicate either determination by a recessive chromosomal gene *or* cytoplasmic inheritance with low transmission of the E^r allele. Out of 64 mutants selected on 1 mg erythromycin/ml, all were stable and 59 showed cytoplasmic inheritance (5 were not tested). Eighteen of these cytoplasmic E^r mutants were chosen at random and all were shown to lose the ability to transmit the E^r genotype in crosses after undergoing mutation from $\rho+$ to $\rho-$, indicating that these E^r mutations had occurred in mitochondrial DNA. Finally, one E^s and one E^r diploid clone segregating from the progeny of one of the D6 $E^r \times$ D22 E^s crosses were sporulated; they showed 4 E^s:0 E^r and 0 E^s:4 E^r segregations respectively, as expected.

The NEWCOMBE *experiment:* As it is used here, $0.8–3.0 \times 10^5$ E^s cells are spread on a filter lying on a plate of YEPG. The plates are incubated for 12 to 27 hours, during which time the cells undergo from 3 to 10 cell generations, or 2.8×10^6 to 1.2×10^8 cell divisions. At this time, cells are washed off of some filters and counted; the remaining filters are transferred to YEPG plates containing an inhibitory concentration of erythromycin. On some of these filters, the cells are redistributed by adding a few drops of water and rubbing with a sterile glass rod. If E^r mutant cells arise before exposure to erythromycin the ratio of mutant cells (colonies on respread plates) to mutant clones (colonies on undisturbed plates) is given by $r/a = t$, where t is the number of generations of prior growth on YEPG (see equations 4 and 6a, LURIA and DELBRÜCK 1943). This result, which is obtained with most bacterial mutants, was obtained in the present experiments with *control* plates on which from 5 to 40 established E^r cells were plated with about 10^5 E^s cells. As shown in Figures 1 and 3, the expected number (5 to 40) of colonies appeared after 42 to 62 hours on the unspread plates; on the respread plates, a great excess of colonies appeared, consistent with the number of generations of prior growth on YEPG. These control experiments show that (1) the respreading procedure is effective, and (2) E^r cells reproduce at essentially normal rates on YEPG in the presence of a great excess of E^s cells.

On the *experimental* plates, the opposite result was obtained. Resistant colonies appeared on the respread and unspread plates at precisely the same rate and beginning at the same time in some experiments. In some cases, the rate was slightly higher on unspread plates (Figure 2). In other cases, the rate was slightly but significantly higher (Figure 1), and the lag slightly reduced but still obvious,

in the respread plates. In one experiment, a burst of about 500 colonies appeared on a single respread plate at the same time that the established E^r cells formed visible colonies on the control plates. Tests showed that most or all of the colonies were composed of mitochondrial mutants. They were probably the progeny of a single E^r cell which preexisted in the population, or which arose early during the nine generations of growth before respreading.

The results of the NEWCOMBE experiment as described above were obtained, with no apparent differences, using stocks D6 and D22. The lag in appearance of new E^r mutants on selective plates has also been observed with a variety of other stocks. Buffering the medium or changing the type of filter did not change the results. The NEWCOMBE experiment was performed once using E^s and E^r *diploids* obtained from crosses of D22 × D6, with the same results. Finally, it was performed once for C^r mutants, using stocks 41 and 41 C^r_{30}; the results showed that most new C^r mutant cells obtained on plates of YEPG plus chloramphenicol arise only *after* exposure to the selective agent, as in the case of E^r mutant cells.

Attempts to distinguish between intracellular selection and mutation induction: In an attempt to rule out the possibility that erythromycin might actually be inducing E^r mutations in large numbers of mitochondria in single cells, two experiments were performed. In the first, cells were grown for 14 to 18 generations on YEPD or on YEPD plus erythromycin. On a fermentable substrate such as the dextrose in YEPD, the mitochondrial genome is not required for cell and mitochondrial growth, as shown by the growth of $\rho-$ mutants which completely lack information-bearing mitochondrial DNA but retain and reproduce mitochondria with poorly-developed cristae and deficient in certain enzymes (MEHROTRA and MAHLER 1968; NAGLEY and LINNANE 1970). Under these conditions it is possible that intracellular selection based on mitochondrial genotype would be reduced or eleiminated, while a hypothetical induction of mutations would continue. On the induction hypothesis, mutant cells would accumulate during this period and be detected when the cells were subsequently replated on YEPG plus erythromycin; no such accumulation would occur on the selection hypothesis. The latter result was obtained (Figure 3).

In the second experiment, filters with cells were grown for 6, 18, or 42 hours on YEPG or YEPG plus erythromycin and chloramphenicol, and then transferred to YEPG plus erythromycin alone. During the pregrowth period, mutation induction should occur if it can, while there should be no selection for E^r mitochondria (most spontaneous E^r mitochondria will remain C^s and be inhibited by chloramphenicol). The filters on YEPG plus erythromycin (Figure 4) showed no indication of accumulation of E^r mutants during the pre-growth period.

DISCUSSION

Mutation rates: The results of the NEWCOMBE experiment show that most E^r mutant cells arise *after* E^s cells are exposed to erythromycin. In the experiments done with clone D6, a total of more than 3.1×10^8 E^s cells on filters were exposed

to erythromycin and spread; no plates carried any E^r mutant cells at this point, so that the frequency of mutant cells in a log phase population of D6 E^s cells is less than $3.7/3.1 \times 10^8 = 1.2 \times 10^{-8}$ (3.7 is the 95% confidence limit of the value of the zero term of a Poisson distribution). These cells resulted from a total of more than 3.1×10^8 cell divisions in the absence of the antibiotic; the combined nuclear and mitochondrial mutation rate from E^s to E^r cells is thus less than $3.7 \ln 2/3.1 \times 10^8 = 8.3 \times 10^{-9}$ mutations/cell division. In clone D22, a single mitochondrial mutant was found which possibly arose early in the course of log growth on YEPG, indicating a mutation rate of 5.5×10^{-10} mutations/cell division. In marked contrast, on YEPG plus erythromycin new E^r mutants arose at rates of about 1 E^r cell per 5×10^7 E^s cells per day. Most of these mutants are mitochondrial when selected on 0.1 or 1 mg erythromycin/ml; this is in agreement with the results of THOMAS (personal communication), who found nuclear E^r mutants only at lower drug concentration.

Possible mechanisms: Three possible explanations must be considered for the origin of mutant cells after exposure to the selective agent.

1. *Intercellular selection,* favoring cells with some E^r mitochondria in the presence of erythromycin and favoring pure E^s cells in its absence. This possibility arises because some residual cell division does occur after cells are exposed to erythromycin. The bulk of this cell division was shown to be completed by the end of the first two days, and is probably limited to the first day (PERLMAN and MAHLER 1970). If the appearance of E^r cells resulted entirely from intercellular selection during this period, then mutant colonies should have appeared primarily in a burst about two days later. Also, one must explain why few or no pure E^r cells arise during the extensive growth on YEPG prior to exposure to antibiotic. The control experiments with artificial mixtures of E^s and established E^r cells rule out stringent intercellular selection in this case. However, this does not mean than there is no intercellular selection whatsoever; it may contribute somewhat to the origin of mutant cells, and certainly is operating *after* these are formed.

2. *Mutation induction* by the antibiotic. This is *a priori* unlikely: it is probable that, in yeast as in bacteria, all effects of chloramphenicol and erythromycin stem from their inhibition of translation. If this resulted in enhanced mutation rates, e.g. due to inhibition of synthesis of DNA repair enzymes, then the induction of mutation should be nonspecific. But data presented in this paper indicate that E^r mutations are not induced by a mixture of chloramphenicol and erythromycin. Moreover, SLONIMSKI (personal communication) has shown by a different method that erythromycin does not induce C^r mutations. It is known that erythromycin and chloramphenicol induce mitochondrial ρ^- mutations (WILLIAMSON, MAROUDAS and WILKIE 1971), but this effect occurs in cells grown for many generations on glucose, where E^r mutants are not produced.

3. *Intracellular selection* favoring E^r mitochondria in mixed cells in the presence, or E^s mitochondria in the absence, of erythromycin. Such selection would require that some product(s) of mitochondrial protein synthesis *not* be shared equally among all mitochondria. The hypothesis also requires that cells exposed

to erythromycin and containing predominantly E^s mitochondria either be capable of a low rate of metabolism and of synthesis of essential mitochondrial components made outside mitochondria, or that such cells contain a pool of those components sufficient to permit some growth and division in an E^r mitochondrion. The production of E^r cells should be largely or entirely eliminated when the mitochondrial genome is rendered nonessential by dextrose fermination or nontranslatable by chloramphenicol; this prediction has been verified. The observed kinetics of appearance of new E^r mutant cells are probably a complex function of the distribution of preexisting E^r mitochondria among the cells, the rate of occurrence of new mitochondrial mutations, and the time required for selection to transform E^s into E^r cells.

Intracellular selection of mitochondria has also been demonstrated in exconjugant paramecium cells containing various mixtures of E^r, E^s, C^r, and C^s mitochondria (ADOUTTE and BEISSON 1972; BEALE, KNOWLES and TAIT 1972). In the case of yeast, THOMAS and WILKIE (1968) invoked intracellular selection to explain why zygotes from a cross $E^r \times E^s$ produced only E^r progeny when plated on YEPG plus erythromycin. However, the postulated selection occurs even in glucose-grown cells (THOMAS 1969); this, together with the data of COEN *et al.* (1970), suggests that other factors may be involved. Intracellular selection is one of several hypotheses which have been suggested to explain the suppressivity of some $\rho-$ mutants in crosses with $\rho+$ (CARNEVALI, MORPURGO and TECCE 1969; EPHRUSSI, JAKOB and GRANDCHAMP 1966; COEN *et al.* 1970). The NEWCOMBE respreading experiment and the LURIA-DELBRÜCK fluctuation test have been applied to Chlamydomonas (SAGER 1962; GILLHAM and LEVINE 1962), showing that the streptomycin-resistant *sr-500* mutant cells arise *after* exposure to streptomycin. It is not yet clear whether the mutations occur in plastid, mitochondrial, or other nonchromosomal DNA. These results have been interpreted in terms of mutation induction (SAGER) as well as intracellular selection (GILLHAM and LEVINE).

The results described here, and those reported for Paramecium and Chlaymdomonas, are encouraging for the student of mitochondrial genetics because they suggest that the multiplicity of mitochondrial genomes within a single cell will not make it unduly difficult to obtain mitochondrial mutants in higher organisms. Although a single mutant mitochondrion would generally be undetectable among a population of hundreds or thousands of wild-type mitochondria, intracellular selection can quickly produce cells in which the mutant mitochondrion is in the majority and can be detected by its effects on the organism's phenotype. This same consideration has obvious implications for mitochondrial evolution and for the role of the mitochondrial genome in the evolution of organisms. It is interesting to note that intracellular selection provides an orthodox mechanism, in terms of spontaneous, random mutation and selection at the organelle level, for the heterodox concept of direct adaptation at the cell level.

This paper is dedicated to PROFESSOR T. M. SONNEBORN on the occasion of his 68th birthday and the 36th anniversary of his discovery of mating types in Paramecium, as a very small token of my gratitude for his friendship, instruction, and example.

I wish to thank DAVID WILKIE for generously providing instruction and facilities for research with yeast during my stay at University College, and for his encouragement and interest during the initiation of this project. Insightful and constructive criticism of the manuscript was supplied by BRUCE GRIFFING, PHILIP PERLMAN, JOHN R. PREER, JR., and DAVID WILKIE.

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