ETHIDIUM BROMIDE INDUCED MUTATION OF YEAST MITOCHONDRIA : COMPLETE TRANSFORMATION OF CELLS INTO RESPIRATORY DEFICIENT NON-CHROMOSOMAL "PETITES".

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It is well known that the intercalating acridine dyes induce mutants in yeast characterized by a respiratory deficiency of the mitochondria and by a non-chromosomal mode of inheritance (Ephrussi, 1952 ; Slonimski, 1953). Recently it has been shown that these mutants, which are called $\rho-$ "petites", result from a change in the buoyant density of their mitochondrial DNA (Mounolou, Jakob and Slonimski, 1966, 1967).

The following properties characterize the process of induction by acridines : a) Induction requires active growth and cell multiplication (Ephrussi *et al*, 1949). b) Mutation rate is close to 1 if defined as the probability of a bud taken at random giving rise to a mutant clone. This means that all of the cells newly born in the presence of acridine are mutated. However, the original input of $\rho+$ "grande" cells does not decrease and may even increase slightly in number (it does, of course, decrease in proportion). After withdrawal of the dye these cells breed true as "grandes". *The mother cells do not mutate* (Ephrussi and Hottinguer, 1951 ; Marcovich, 1951). c) The activity spectrum of various acridines as $\rho-$ mutagenic agents is very similar to the one deduced from the study of their inhibitory action on the induced biosynthesis of cytochrome oxidase in non-growing cells (Slonimski, 1953).

Ethidium bromide (abbreviated EB), another intercalating dye which has been extensively studied by Waring (1966) and le Pecq (1965) has recently been shown to possess interesting properties with respect to covalently closed duplex DNA. It modifies the superhelical structure and permits the separation of circular and non-circular molecules (Radloff *et al*, 1967). In the case of yeast and *Neurospora* the question of circularity of mitochondrial DNA is still open (see discussion in the Bari Symposium, 1967) and it is not known whether the isolation of non-circular molecules results from the action of nucleases or reflects a fundamental difference between fungi and higher organisms. It was therefore of interest to see whether EB can induce $\rho-$ non-chromosomal mutants in yeast and to compare its action with that of acridines.

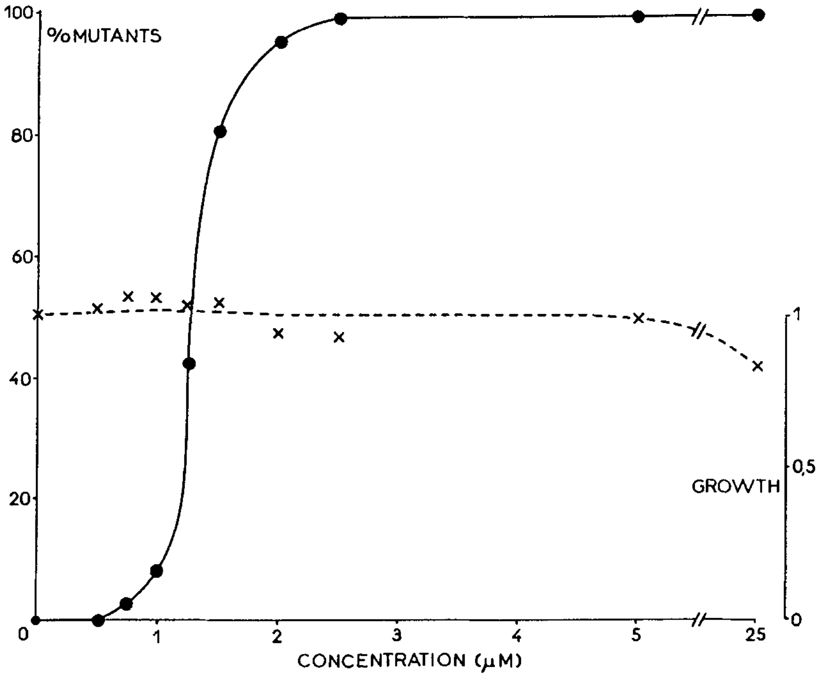


Fig. 1. - Mutagenesis as a function of ethidium bromide concentration : "Grande" $\rho+$ strain "Yeast Foam" was grown aerobically at 25°C in a synthetic medium G (Galzy and Slonimski, 1957) with 0.1M glucose as carbon source. Cells were harvested during the exponential growth phase (10^7 cells per ml) and inoculated at a density of 10^6 cells per ml into a fresh medium of the same composition with 0.1M phosphate buffer (pH 6.5) and containing various concentrations of EB. After 7 hours of aerobic growth at 25°C the cells were washed, diluted and plated on drug free medium containing 2% glycerol, 0.1% glucose, 1% yeast extract and 1% bacto peptone. The numbers of small size colonie ($\rho-$ clones) and normal size ($\rho+$ clones) were counted after incubation at 28°C for 4 days. The ordinate give : (a) the % of $\rho-$ clones in the total number ($\rho-$ and $\rho+$) of clones counted ; (b) the relative growth normalized to 1 for the control which, in the absence of EB, has undergone 3.2 cell divisions.

Fig. 1 shows that EB is an extremely efficient mutagen. After the culture has undergone 3,2 doublings in its presence more than 99,8% of the cells are "petites". This value is much higher than that which can be obtained under identical conditions by the most efficient acridine, 2,8-diamino-N-methyl acridine (Slonimski and de Robichon-Szulmajster, 1957). It is interesting to find that the frequency of mutants as a function of EB concentration is not linear, but S-shaped. This suggests that mutagenesis is a cooperative process ; i.e., either that two or more molecules of EB are required simultaneously to produce a mutational event, or that we are dealing here with a threshold phenomenon involving two or more targets per cell. A similar, but less pronounced cooperativity has already been observed in the case of euflavine (Slonimski and de Robichon-Szulmajster, 1957). Fig. 1 also shows that concentrations of EB that produce a complete transformation of the whole population into "petites" do not inhibit cell multiplication at all, nor do they produce any mortality. In this respect EB is a more selective mutagen than the acridines. The gap separating the mutagenic concentrations and the generally toxic or growth inhibitory concentrations of the drug (see Marcovich, 1953) is much more pronounced in the case of EB.

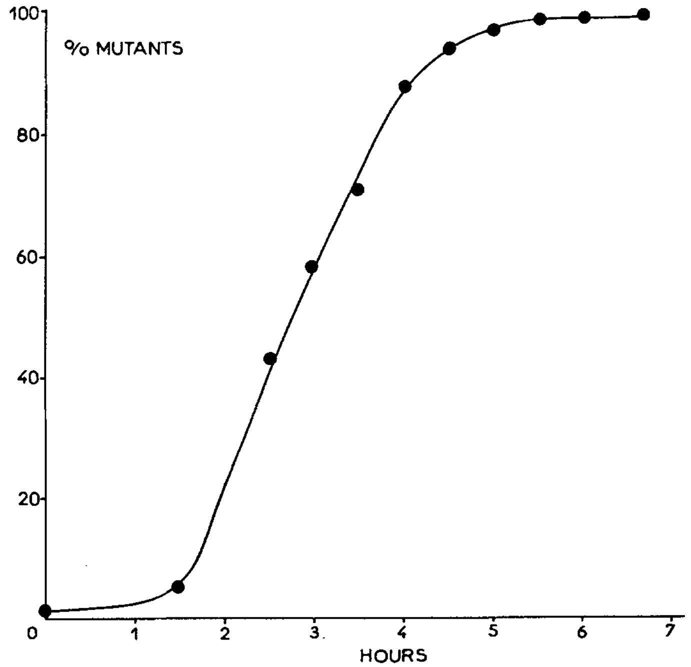
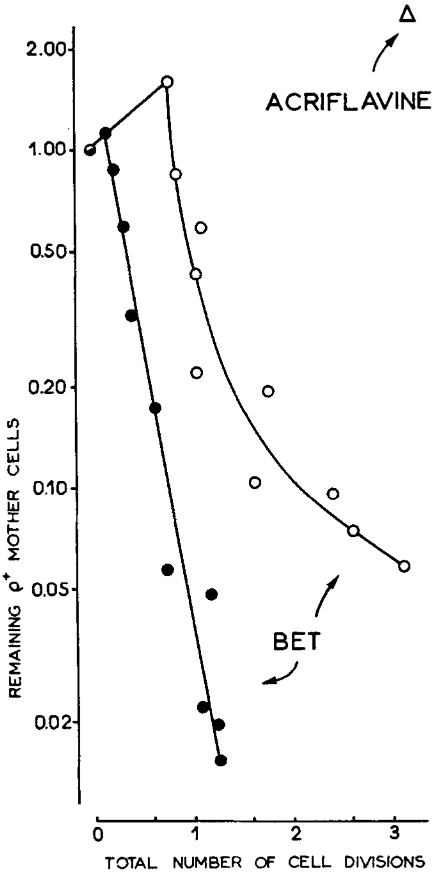


Fig. 2. - Mutagenesis by ethidium bromide as a function of time : Cells were withdrawn after variable time from constant (3.5×10^{-6} M) concentration of EB. Other conditions as for fig. 1.

It has been verified that the "petites" obtained after treatment with EB have identical deficiencies of the cytochrome system as those produced by acridines, i.e. they have no cytochromes ($a + a_3$) and b. Also, they do not grow on non-fermentable energy sources and do not respire. "Petites" induced by ethidium in haploid strains do not complement when crossed with the reference ρ- strain, C982-19dA$_1$.

The time course of the appearance of ρ- mutants is given in fig. 2. One passes from less than 5% of mutants to more than 90% in about 2,5 hours, i.e., a time interval corresponding to one cell division.

The most striking effect of EB concerns its action on the mother cells. It was mentioned earlier that the original input of ρ+ cells is not readily transformed into "petites" by acridines. Fig. 3 shows the results of a compa-



<u>Fig. 3.</u> - <u>Transformation by ethidium bromide of</u> ρ+ <u>mother cells into</u> ρ- <u>mutant cells under growing conditions</u> :

Experimental conditions during mutagenesis were as for fig. 2

Abscissa : number of cell divisions undergone based on the sum of ρ+ and ρ- cells.

Ordinates : number of ρ+ cells per ml. (normalized to 1 = 10^6 cells per ml, i.e., the initial number) remaining after growth in EB (circles) or 3 x 10^{-6}M acriflavine (B.D.H., triangle). Open circles correspond to a culture which was grown with glucose as carbon source (as for fig. 1) before treatment with EB. Full circles correspond to a culture which was grown in a similar way but with 0.2M Ethanol as carbon source before treatment with EB. In all cases glucose was the carbon source during the EB treatment.

rison of the effects of acriflavine and EB on the transformation of $\rho+$ mother cells into "petites" under growing conditions. A great difference between the two drugs is evident. In acriflavine, the number of $\rho+$ cells in the culture has increased some 2,5 times the original number. By contrast, in the case of EB there is a rapid transformation of the mother cells into "petites".

In this experiment the cells which were treated with EB had first been grown under either of two culture conditions. One culture had first been grown with glucose as the energy source and harvested during the exponential phase of growth. In this case glucose repression occurs and the cells will have only rare and poorly developed mitochondria and will be deficient in several respiratory enzymes. In the second culture ethanol was used as the energy source. This culture will be respiring actively and the cells will have both fully developed mitochondria and a full complement of respiratory enzymes (cf. Ephrussi *et al*, 1956 ; Yotsuyanagi, 1962). It is apparent from fig. 3 that the mother cells with numerous, fully developed mitochondria are transformed by EB at least as readily as mother cells that have only few mitochondria. An opposite result would be expected if there was a direct relationship between the number of mitochondria per cell and the resistance to be transformed by the dye.

The transformation of the mother cells from $\rho+$ to $\rho-$ is most clearly shown when the effect of EB is examined under non-growing conditions. In this experiment, where cells were subjected to starvation conditions before being suspended in EB in buffer solution only, the total number of cells increased by only 21% in 36 hours. However, complete transformation of the cell population from "grande" to "petite" occured over a short time within this period. This proves that EB, unlike acridine, transforms the mother cells themselves and not only the newly born cells are mutant.

As can be seen in fig. 4 the transformation of non-growing $\rho+$ cells in $\rho-$ mutants follows *simple first order kinetics* (with a half life of ca. 2 hours) *as a function of time* after an initial period during which there is no detectable mutagenesis. A classical interpretation of this type of kinetics will be the following : a $\rho+$ cell has a given number of independent and autonomous targets with which EB can combine and mutate ; a cell which has at least *one free target* (i.e., one which has not reacted with EB) will still give rise to a $\rho+$ clone, but if all the targets have reacted the cell will give rise to a $\rho-$ clone. The number of targets per cell can be estimated from the intercept of the first-order part of the curve and was found to be not greater than 6 in the experiment shown in figure 4, and other experiments gave similar figures.

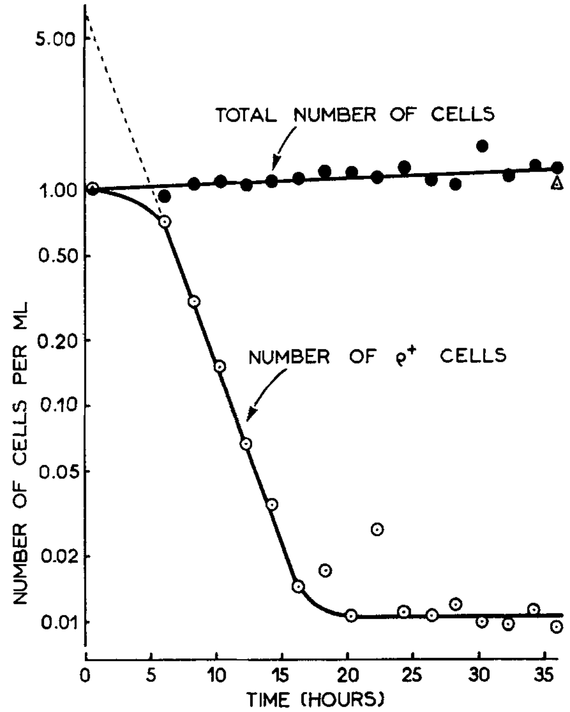


Fig. 4. - Transformation by ethidium bromide of ρ+ mother cells into ρ- mutant cells under non-growing conditions :
Cells were first cultured with 0.2M ethanol as carbon source and were then incubated for 20 hours in 0.1M buffer containing 0.1M glucose. They were then suspended in buffer with no carbon or nitrogen source but containing a constant concentration (3.5×10^{-6}M) of EB and withdrawn after variable time.
The ordinates give : (a) number of ρ+ cells per ml remaining after suspension in EB for varying periods of time (open circles), and (b) total number of cells (ρ+ and ρ-) per ml. (full circles). The triangle represents the total number of cells per ml. and, on this scale, also the number of ρ+ cells per ml. remaining after 36 hours in a control without EB. In all cases the numbers of cells per ml. have been normalized so that 1 = 1.4×10^6 cells per ml., i.e., the initial number.

It is tempting to equate the targets with the genetic material of mitochondria, i.e. mitochondrial DNA, or with the organelles themselves.
If this is done, one has to face a discrepancy in numbers. Although the estimation of the number of targets per cell may be suffering from the well known difficulties of the target theory it is apparent, from our experiments, that this number is relatively small. Now, the number of molecules of mitochondrial DNA per cell is not known precisely, but it is safe to say that it is greater than 6 by, at least, one order of magnitude. This discrepancy could be interpreted by assuming that not all of the molecules of mitochondrial DNA are

autonomous replicating units. Instead, only a fraction of them plays a key role in the hereditary transmission of the respiratory enzymes and it is this key fraction which constitutes the actual targets of EB. The same arguments can be applied if we consider the mitochondria themselves to be targets for EB. We are not certain of the precise number of mitochondria per cell but, especially in the case of the diploid, it is probably greater than our estimate of 6 targets per cell (see Avers et al, 1965). Furthermore, if all the mitochondria are autonomous replicating units and targets for EB we should have found that $\rho+$ mother cells first cultured on ethanol would be transformed to $\rho-$ less rapidly than those first cultured on glucose whereas, if there was any difference, the reverse was in fact found (see fig. 3).

Whatever the answer to the problem of non-equivalence and autonomy of mitochondrial DNA molecules and/or of the organelles themselves could be, it is established that :

a) ethidium bromide is the most specific and potent agent inducing $\rho-$ mutants ;
b) it acts in the absence of cell multiplication and transforms, following first-order kinetics, all of the mother cells into mutants. This suggests that mitochondrial DNA in yeast may be natively in the form of superhelical circles and that the changes in the supercoiling after combination with the dye initiate the mitochondrial mutation.

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