MITOCHONDRIAL MEMBRANES AND MUTAGENESIS BY ETHIDIUM BROMIDE

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The conversion of wild type (ρ^+) to cytoplasmic *petites* (ρ^-) in *Saccharomyces cerevisiae*, à mutation in mitochondrial DNA, can be brought about with high efficiency by low concentrations of ethidium bromide (EB). The rate and extent of mutagenesis and its expression can be influenced, and even reversed, by a number of genetic lesions, agents or treatments affecting mitochondrial structure and metabolism. Among them are incubation at 45°, exposure to Antimycin A, growth on different carbon sources and the presence or absence of 2 different gene products previously implicated in the repair of UV induced lesions in mitochondrial DNA. Based on these observations a model for EB mutagenesis is advanced which postulates a complex between mitochondrial DNA and the inner membrane as the target susceptible to modification by EB. This model predicts that altered membranes should lead to changes in the susceptibility of cells to the mutagenic action of EB. This prediction has been verified by comparing cells that contain one of 2 structurally quite distinct monounsaturated C_{18} fatty acids in their mitochondrial phospholipids: greater resistance to mutagenesis and ease of thermal protection is exhibited when cells — and mitochondria — contain oleic ($\Delta^9 cis$, m.p. <5°) rather than petroselinic ($\Delta^6 cis$, m.p. 28°) acid in their phospholipids. As a corollary, studies on EB mutagenesis and mitochondrial DNA may be used as probes for the mitochondrial inner membrane to reveal some perhaps novel functions.

1. INTRODUCTION

The phenanthridinium dye ethidium bromide (EB) is finding increasing use as a most versatile probe for various aspects of the structure and function of extrachromosomal genomes (1-8). When added to yeast cells of various species, for instance, the dye at low concentrations has been shown to be capable of selectively interfering with the duplication (9-12), transcription (13,14) and eventual expression of mitochondrial DNA (mtDNA) (12,15-18). Among its most striking effects — exerted on facultative anaerobic yeast such as *Saccharomyces cerevisiae* — is its ability to induce the cytoplasmic *petite* (ρ^-) mutation in all the cells of a population (16,19). In this regard, as well as its mutagenicity in the complete absence of cellular, or mitochondrial, growth and division, it appears to differ fundamentally from the superficially

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similar action of other intercalating molecules such as the acridines (20,21). The molecular correlate of the formation of mtDNA-containing ρ^- cells in a population is the gradual disappearance of parental mtDNA and its replacement by a collection of progeny molecules, heterogeneous in size, buoyant density and content of genetic markers (9,10,22,23).

It is the purpose of this report to demonstrate that certain aspects of this mutagenesis by EB appear to be susceptible to a variety of subtle alterations in cellular and mitochondrial phenotype and genotype, not readily interpretable in terms of alterations exclusively in mtDNA. In consequence, it seems reasonable to postulate a complex between mtDNA and the inner mitochondrial membrane as the initial target for interaction with EB. As a correlate, studies on EB mutagenesis may reveal hitherto unanticipated functions of the mitochondrial inner membrane.

2. METHODS

(a) *Strains and growth conditions*

The following strains were used in these studies.

Strain	Ploidy	Genotype		Source	Remarks
		nuclear	mitochondrial		
Fleischmann	diploid		ρ^+	commercial	standard strain
N123	haploid	αhis_1^-	ρ^+	E. Moustacchi	standard for uvs strains
uvs ρ5	haploid	αhis_1^- (ρ5)	ρ^+	E. Moustacchi	chromosomal uvs ρ
uvs ρ72	haploid	αhis_1^-	ρ^+ (ρ72)	E. Moustacchi	may be mitochondrial
KD 115	haploid	*ole*-1-2	ρ^+	A. Keith	desaturase negative
IL-8-8C	haploid	$\alpha his^- try^-$	$\rho^+ C^R E^R$	P. Slonimski	CAP,Erythro resistant

All strains were cultured at 30° with aeration on semisynthetic media (18,24) supplemented with *his* or fatty acids, as required, with 1 or 5% glucose, 3% lactate, or 3% glycerol as carbon source. All cells were always in early to mid-exponential phase ($<10^7$ per ml); thus their level of respiratory enzymes was fully repressed when grown on glucose, and lactate and glycerol grown cells were fully derepressed. These 2 culture conditions yield cells which are distinctly different morphologically and biochemically. With respect to mutagenesis by EB and related manipulations they are qualitatively but not quantitatively similar.

(b) *Plating conditions*

Cell number and the proportion of petites in a population were measured by plating a suitably diluted aliquot of cells on solid media (2% agar) containing 0.1 or 1% glucose, 3% lactate, 3% glycerol or 0.1% glucose plus 3% lactate or glycerol. The proportion of ρ^+ cells was measured in different ways depending on the carbon source present in the agar: 1) 1% glucose — tetrazolium (TTZ) overlay procedure of Ogur *et al.* (25) which causes ρ^+ cells to turn red while ρ^- remain white; 2) 0.1% glucose plus 3% lactate or glycerol —

ρ^- cells form small colonies while ρ^+ cells form large ones; 3) 3% lactate or 3% glycerol — only ρ^+ cells form colonies. In this last case the fraction of ρ^+ colonies may be estimated from the ratio: colonies on 3% lactate / colonies on 1% glucose.

On 1% glucose-TTZ plates, sectored colonies (part red and part white) were observed; these were scored as ρ^+. In general, sectored colonies were rare (<15% of ρ^+) when ρ^+ = 15 to 99%. When the percentage of ρ^+ cells was low, sectored cells usually accounted for more than 50% of the ρ^+ colonies; it has been proposed that such colonies result from drug-induced genetically intermediate cells. Values shown are means of duplicate or triplicate platings.

Solutions of EB and acriflavin were prepared fresh daily and used in foil wrapped containers. The EB concentration was calculated for each sample based on the absorbance at 480 nm with a millimolar extinction coefficient of ε_{mM} = 5.65.

(c) *DNA experiments*

Studies of the effects of EB on mtDNA were done essentially as described previously (10). Cells were labeled in the presence of cycloheximide (CH) which causes a cessation of nuclear DNA (nDNA) synthesis without affecting mtDNA synthesis; in this way mtDNA was preferentially labeled and thus could be studied in extracts of whole cells. Specific details are presented in the figure legends.

(d) *Materials*

Acriflavin, antimycin A, chloramphenicol, cycloheximide, ethidium bromide and triphenyl-tetrazolium chloride were purchased from Sigma Chemical Co. CsCl was obtained from Harshaw Chemical Co.; 8[^{14}C] and [^{3}H]adenine (52.6 mCi per mmole and 23.4 Ci per mmole respectively) were obtained from New England Nuclear. Erythromycin glucoheptonate was kindly donated by Eli Lilly Co. Oleic and petroselinic acid (≥99% pure) were purchased from the Hormel Institute, Austin, Minn.

3. RESULTS

(a) *Initial observations and definitions*

These studies had their inception with our discovery that EB mutagenesis appeared to involve an initial thermoreversible phase (26). Simultaneously, Slonimski and his collaborators reported that cells could be protected from the action of the mutagen by prior growth on chloramphenicol, or exposure to acriflavin (27). In parallel investigations our two groups showed that the mtDNA of certain petites which had retained antibiotic resistance markers, appeared more resistant to the destructive action of EB (23). We thus can envisage 3 possible modes in which some treatments or predisposition of a cell population can influence its susceptibility to mutagenesis by EB. We shall speak of *protection* when the agent is added, or treatment performed, prior to mutagenesis; of *competition* when it and mutagenesis are concurrent, and of *reversal* or *cure* when the second agent is added only after the mutagenic exposure has been terminated by the removal of all extracellular EB.

(b) *Alterations of phenotype*

Heat. We have published observations dealing with the reversal by heating of lactate-grown diploid cells, mutagenized under nongrowing conditions (starvation and EB treatment in phosphate buffer) (26). The most characteristic feature was the sharp temperature dependence of the effects, suggesting some co-

operative phenomenon as its cause. These studies have now been extended to a number of different strains, haploid as well as diploid, grown and mutagenized under a variety of different conditions. Qualitatively, an increase in ρ^+ progeny after heating such EB induced cells (EBIC) in the range of 42 to 45° was observed in all instances, although the precise quantitative parameters remain to be established.

To test whether the thermal reversal might be critically dependent on the denaturation of a macromolecule(s) we next asked whether preheating the cells to these temperatures affected the subsequent rate of mutagenesis. In a control experiment we first established that such heat treatment had no effect either on intramitochondrial (cytochrome oxidase, NADH oxidase, and L-malate dehydrogenase) and extramitochondrial (L-malate and L-glutamate dehydrogenases) enzymes. As can be seen from the data in Table 1, exposing our standard, lactate-grown diploid cells to heating for 1 hr in buffer exerted a significant protection against subsequent mutagenesis by EB under either nongrowing or growing conditions; the latter even after 1.5 generations of growth subsequent to the heating step. Thus if heating involves a conformation change of a biopolymer(s) this event must be virtually irreversible and maintain its effectiveness even with macromolecules in the immediate progeny.

Inhibitors of transcription and translation. As mentioned already, exposure to acriflavin and chloramphenicol (CAP) had been reported by Slonimski to lead to protection against mutagenesis. Since it might be difficult to distinguish possible effects by these agents on mitochondrial biosynthesis (12-18,28-30) from ones on mitochondrial function (31-33) we have concentrated on their abi-

TABLE 1

Protection by preheating

Exposure (min) to 25 μM EB	% ρ^+ (red + sectored) remaining					
	Phosphate buffer (0.1 M, pH 6.5) EB 10 min after adding cells		Lactate medium[a] EB 10 min after adding cells		Lactate medium[a] EB 3 hr after adding cells	
	30°	45°	30°	45°	30°	45°
0	100	92	96.8	95	99.4	96.5
20	92		97		96.2	86
40	39		74.4		55.3	
60	9.2	89.3	14.4	83	8.5	86.2
90	3.9		0.9		0.65	
150	2.9	95.1		91.5		82
180		93.4		94		77
240		83		89		51

[a] Generation time ≃ 2 hr.

Diploid cells were grown to a density of 10^7 per ml on 3% lactate medium, washed with 0.1 M phosphate buffer, pH 6.5 (referred to as "buffer") and incubated in buffer at 2 x 10^7 cells per ml for 60 min at either 30 or 45°. Cells were then diluted 20-fold with 30° media as indicated above. EB was added at 25 μM after 10 min or 3 hr of shaking at 30°. Samples were then diluted in cold water and plated at the times indicated on 1% glucose plates (in duplicate).

lity to effect a cure of EBIC. Essentially quantitative reversal can be achieved even with completely repressed cells by incubatin the EBIC with 100 μM acriflavin or 4 mg CAP per ml for 2 hr in buffer at 30°.

Antimycin A. In *S. cerevisiae* Antimycin A is an exceedingly effective inhibitor of respiration (with a blockade at its customary site, i.e. between cytochrome b and c_1) (34), and of all biosynthetic reactions driven by respiration (17,35). On the other hand there is no indication of any other inhibitory site affected by the compound, and it is completely innocuous toward growth or biosynthetic events driven by fermentation, including the early formation of cytochromes and respiratory enzymes consequent to release from catabolite repression (35). It thus appeared of interest to investigate the possible effects of the agent on EB mutagenesis with cells at various physiological states. We found that Antimycin A at exceedingly low concentrations (range from 0.05 to 1.0 μg per ml) was highly active in interfering with EB in all 3 of the modalities tested: protection, competition and cure; it proved capable of exerting all these effects not only with fully derepressed cells (grown on 3% lactate), but also with partially or fully repressed ones (pregrown or growing on 1% or 5% glucose, respectively) of several strains. Some typical experiments with fully derepressed and repressed (35) cells are shown in Figs. 1A and B. The latter results are particularly significant in view of the fact, already mentioned, that under these conditions mitochondrial energy transduction is completely dispensable, and Antimycin A exerts no know effect on macromolecular metabolism. The indication is therefore that whatever the detailed events involved in the labilization and modification of mtDNA involved in mutagenesis, these can be profoundly affected by an agent interacting with certain components of the mitochondrial inner membrane. This hypothesis is strengthened by the observations (see below) that under these conditions Antimycin A does not inhibit the synthesis of either nuclear or mitochondrial DNA. Further confirmation comes from an investigation of the kinetics for the maintenance of the capacity for cure by Antimycin A. As shown in Fig. 2 this capacity appears to be irreversibly lost after exposure of glucose-grown EBIC to complete repressing medium for 90 min: this corresponds rather closely to the times and conditions required to produce extensive breakdown of parental mtDNA (10). It is also of interest that in Fig. 1 the extrapolated zero-time value on the ordinate appears unaffected by the presence of Antimycin; thus whatever the explanation for the initial shoulder, the fundamental mechanism responsible for it must be equally effective in the presence of Antimycin and in its absence. In contrast, oligomycin, another inhibitor of mitochondrial function, but interacting with a site involved in the attachment of F_1-ATPase to the inner membrane (36,37), was completely without effect.

Effects on DNA synthesis. A relatively trivial explanation for the various protective and competitive treatments and agents, applicable in a somewhat modified form also to reversal, would be simply to postulate that EB has been rendered operationally ineffective. A simple mechanism, in the former case, would be that cells or mitochondria have been rendered impermeable to the agent, and in the latter, that it had been detached from its normal site of action. If either of these explanations were correct they would predict a release from the block of mtDNA synthesis ordinarily exerted by EB (9-11). This prediction has been checked explicitly with cells grown on either glucose or lactate, both in the presence of the mutagen, or immediately subsequent to its removal. The inhibition of mtDNA synthesis was not relieved by prior heating of cells; by their exposure to acriflavin; by adding Antimycin simultaneously with EB, or after reversal of a transient mutagenic exposure to EB; or by exposing EBIC to acriflavin, Antimycin A or heat in the absence of growth.

Metabolic state. Earlier observations suggested that the metabolic state of cells (and their mitochondria) appeared capable of influencing the overall ef-

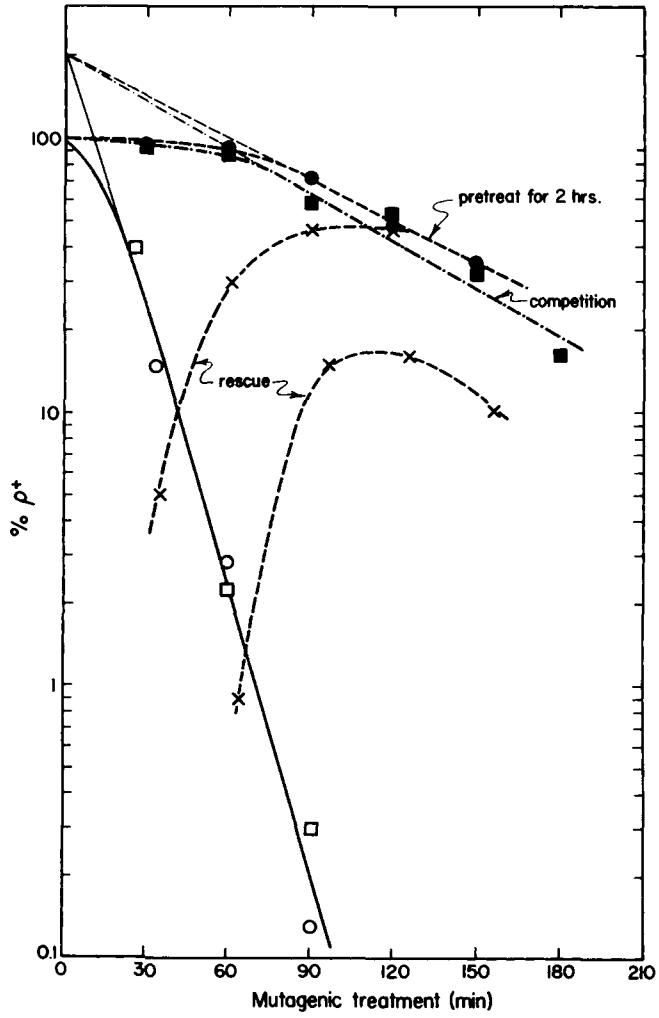

Fig. 1. A: Effects of Antimycin A (1 µg per ml) on starved diploid cells, pregrown on 3% lactate, at a cell density of 2 x 10^6 per ml. Cells were treated with 25 µM EB and plated periodically (O and □ indicate 2 separate experiments). In one sample Antimycin A (Anti A) was present along with EB (competition — ■) and in another, cells were pretreated with Anti A for 2 hr and treated with EB after washing (●). At 35 and 65 min of EB treatment (no Anti A) aliquots were removed, filtered, washed, suspended in buffer with Anti A and plated at intervals as indicated. All platings were on 1% glucose medium.

B: Effects of Antimycin A (1 µg per ml) on starved diploid cells, previously grown on 1% and 5% glucose. Design of the experiments was similar to that shown in Fig. 1A except for the carbon source; competition with, and rescue by Anti A were the parameters tested.

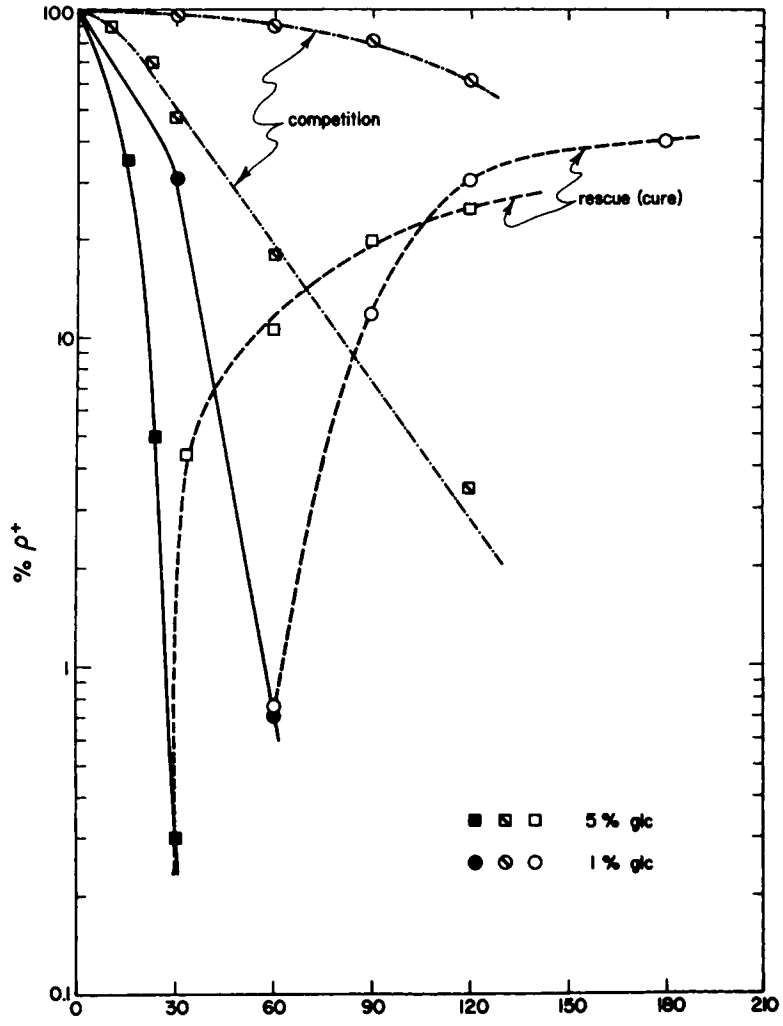

Fig. 1 B.

fectiveness of EB mutagenesis; under identical conditions of cell and EB concentration this effect was preponderantly on the extrapolated slope of the typical semilog plot (e.g. Fig. 1) (16,19). Under the particular conditions used, this rate for lactate grown cells is approximately 0.6 that of cells grown on glucose. However, a much more pronounced influence of carbon source is revealed by a comparison of the different assay systems commonly used for the estimation of mutagenic efficiency on solid medium (38), especially between selective (nonfermentable) and nonselective (fermentable) carbon sources. A representative experiment in terms of the fraction ($1-\rho^-$) of ρ^+ (wild type) survivors is shown in Fig. 3. Although the effect appears quite general, with respect to the nature of the cells used and their prior history, certain specific features do become evident from a consideration of the figure and other experiments not shown here: the effect does not depend on derepressing conditions *per se* (0.1% vs. 1% glucose) but rather on the actual nature of the respiratory carbon source employed — lactate is more efficient than is gly-

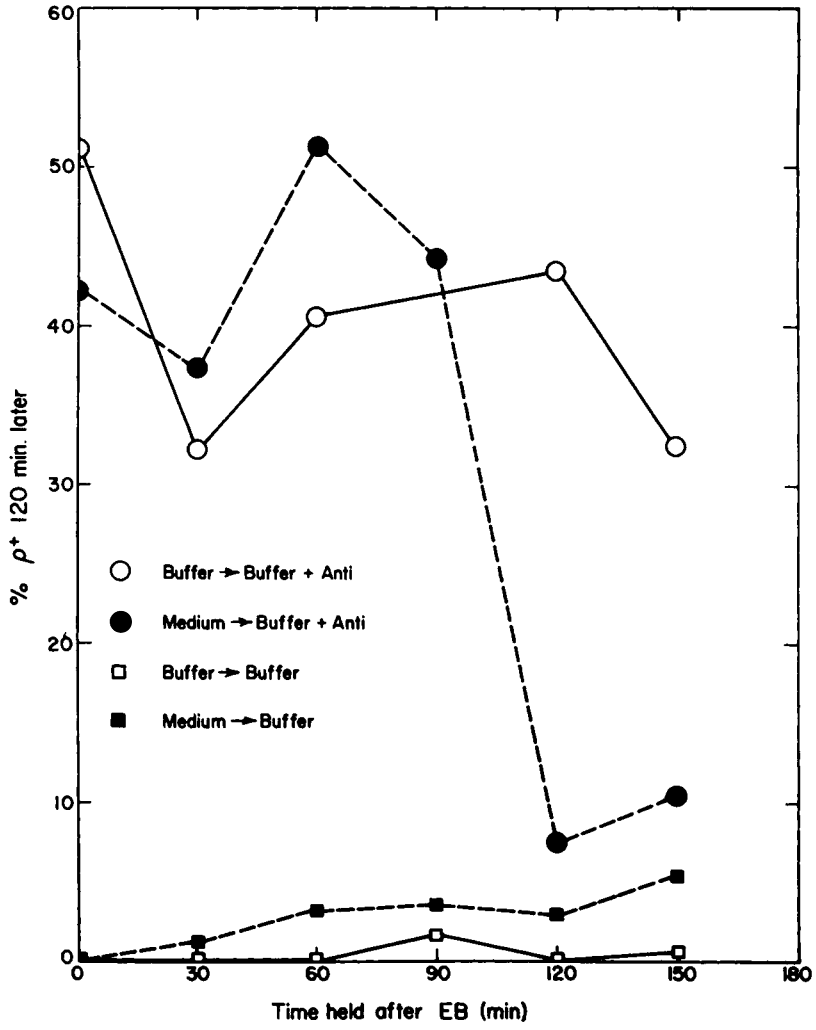

Fig. 2. Decay of capacity for Antimycin reversal. Diploid cells grown to exponential phase on 5% glucose medium were washed, starved in buffer for 60 min and treated with 27 μM EB for 20 min. Cells were then rapidly chilled, washed by centrifugation and suspended at 30° in buffer or 1% glucose medium at a cell density of 2×10^6 per ml. At 30 min intervals for 150 min, cells were diluted 10^{-2} in buffer plus cycloheximide (100 μg per ml) with or without Anti A (1 μg per ml); each diluted sample was then incubated for 120 min at 30° and then further diluted and plated on 1% glucose medium.

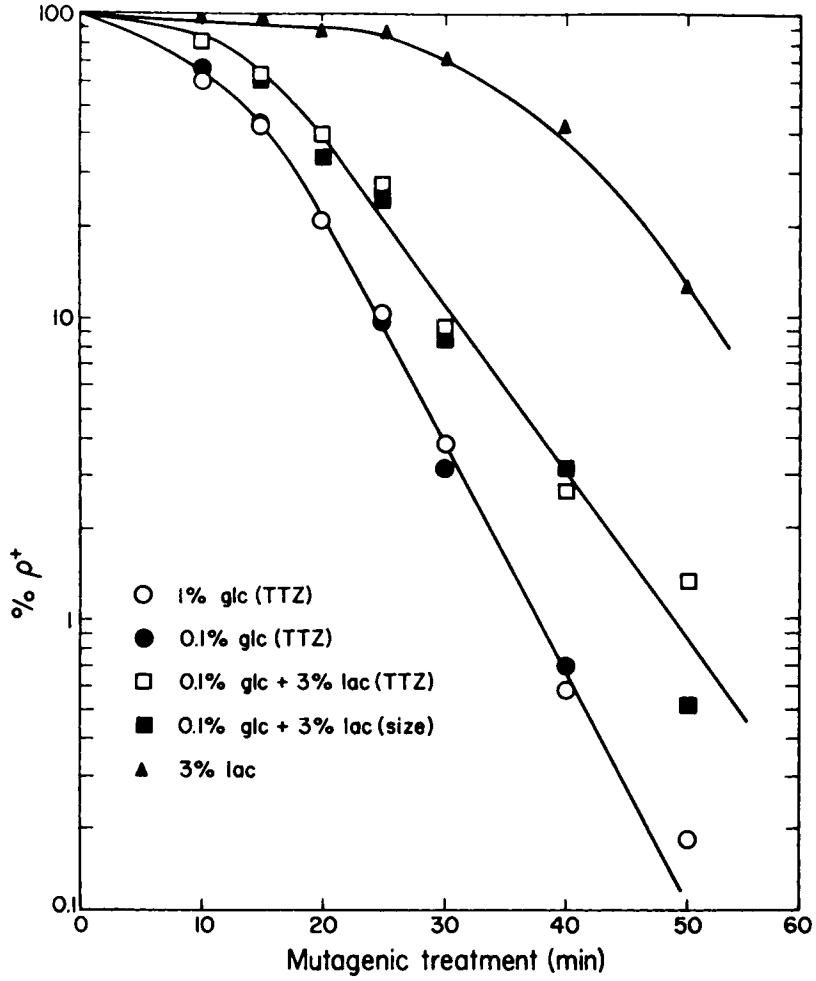

Fig. 3. Effects of plating conditions on EB mutagenesis. Diploid cells grown on 3% lactate medium were washed, starved in buffer for 1 hr and treated with 25 μM EB. At intervals cells were diluted 10^{-3} and 0.1 ml aliquots plated on each of 4 kinds of plates differing only in carbon source. The plates with 0.1% glucose plus 3% lactate were scored for % ρ^+ in 2 ways (TTZ overlay and size difference) which yielded essentially identical results. In a separate experiment it was shown that the mutagenesis curve for cells plated on 1% galactose medium coincided with the 1% glucose curve.

cerol (or ethanol, not shown), which in turn is more effective than the mixture of 3% glycerol-0.1% glucose frequently used in measuring the extent of ρ^- mutagenesis (19).

Further examination of the requirements for this effect which we have termed *metabolic consolidation* (growth on glucose) and *metabolic reversal* (growth on lactate) in liquid culture are shown in Fig. 4 and Tables 2 and 3. As shown in Fig. 4, the initial metastable state of EBIC (pre-cultured in lactate) becomes stabilized after 5 hr of growth on the 2 extreme media at approximately 60% wild type on lactate and 78% mutant on glucose. Examination of the survival of parental mtDNA under analogous conditions, but with a haploid strain (Table 2), shows complete destruction of mtDNA on glucose with a significant retention of this entity on lactate, coincident with the retention of the ρ^+ genome. The data in Table 3 suggest that metabolic consolidation can be observed with cells originally grown either on lactate or glucose, but that metabolic reversal is slower with the latter. They also permit the inference that while both consolidation and reversal are inhibited by cycloheximide and hence probably require cytoplasmic protein synthesis, they appear somewhat less dependent on mitochondrial protein synthesis as evidenced by their relatively greater insensitivity to erythromycin.

(c) *Effects of genotype*

Mutagenesis and mtDNA. In studying the possible effect of EB on a variety of strains, especially those that are already mutant with respect to ρ^+, the very character utilized in the earlier sections of this report, one is restricted to an investigation of possible alterations of mtDNA itself. In wild type cells, transiently subjected to exposure to EB sufficient to convert more than 98% of the cells to ρ^- mutants, one observes the following series of effects (10): 1) The synthesis of mtDNA is blocked as long as EB is present and immediately (for approximately half a generation) after its removal and transfer of the mutagenized populations to growth media. 2) The parental mtDNA is partially degraded during this initial exposure as shown by a) a loss of 30 to 35% of the material banding in the mtDNA peak in either buoyant density or rate sedimentation analysis; b) a greater heterogeneity and smaller size of this mtDNA (Fig. 5). 3) These modifications of parental mtDNA, already initiated in EBIC in the absence of growth, are followed by a very rapid (<2 cell generations) degradation of this entity upon resumption of growth on glucose or galactose. 4) At about this time the synthesis of progeny mtDNA, which first becomes detectable within 0.5 generations of growth, has reached levels comparable to those characteristic of the control. However, the DNA produced in EBIC is of somewhat lower buoyant density and a great deal more heterogeneous than is control mtDNA.

ρ^- *Strains.* Effects of EB on the mtDNA of ρ^- strains are qualitatively different from those of the parent wild type strains. As mentioned in the first part of this section we are investigating this problem jointly with Slonimski and his collaborators (23) and will show elsewhere that EB is still capable of inhibiting the synthesis of ρ^- mtDNA; transient exposure to such treatment does not lead to the rapid destruction of parental templates which therefore remain competent to direct the synthesis of "normal" progeny DNA.

UV sensitive mutants. The increased sensitivity to the lethal (or mutagenic) effects of ultraviolet radiation of certain (UV sensitive or *uvs*) strains has been documented for many organisms, including yeast (39-41). Frequently such sensitivity has been explained in terms of a deficiency in some component of one of the 2 postulated dark repair systems (excision and recombination repair) for such lesions (e.g. 42,43). A particularly interesting class of mutants (*uvs* ρ strains) has recently been isolated by E. Moustacchi (44,45): cells of these strains exhibit normal response to UV exposure by nuclear parameters (lethality and mutation induction) but increased sensitivity to ρ^- formation,

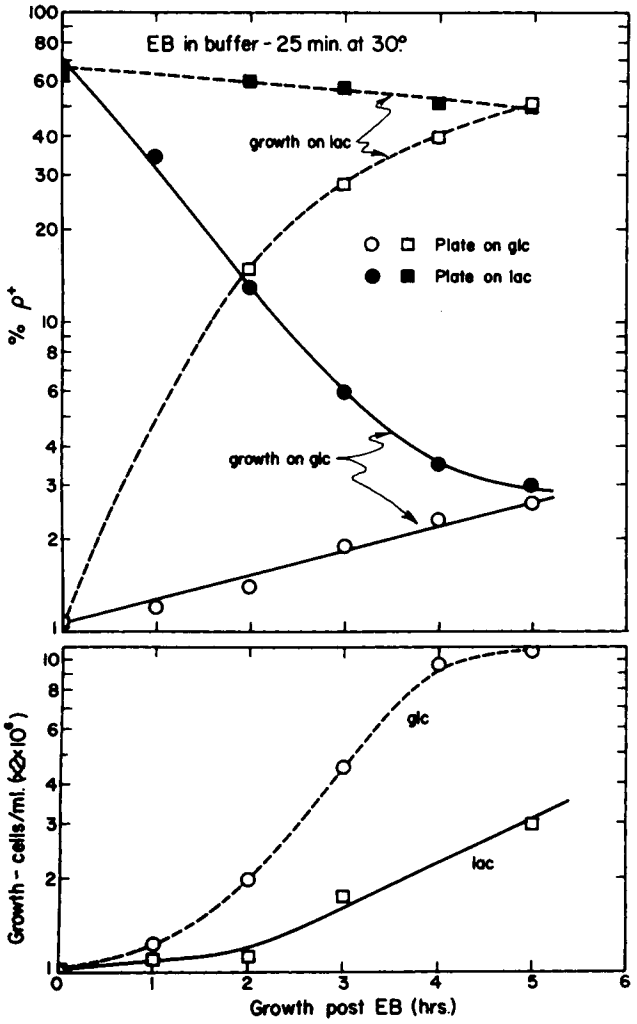

Fig. 4. Metabolic effects on EB mutagenesis. Diploid cells growing on 3% lactate were treated with 25 μM EB for 25 min at 30°. An aliquot was filtered, washed and suspended in medium with 3% lactate or 1% glucose as carbon source; both samples were incubated at 30°. At intervals growth was measured by turbidity and viable count on 1% glucose plates; samples were also plated on 3% lactate. Prolonged exposure to lactate medium is not essential for reversal of EB effects; this suggests that consolidation occurs soon after growth resumes.

TABLE 2

Metabolic effects on mutagenesis and mitochondrial DNA

Gradient	Inital counts retained in mtDNA (%)		% ρ^+ on	
	EBIC (^{14}C)	Control (^{3}H)	1% Glucose (TTZ)	3% Lactose
A. Before EB	(100)[a]	(100)[b]	>99	>99
B. After EB	53	83	<0.2	44
C. Glucose[c]	<8.4	124	0.45	1.05
D. Lactate[c]	31.6	186	33.2	41

[a] 3.9×10^3 dpm.

[b] 1.15×10^6 dpm.

[c] Grown for 2 generations on the indicated carbon source.

Cells of haploid strain IL-8-8C were grown on a semi-synthetic medium containing *his* and *try*, with 3% lactate as carbon source. Labeling of 2 batches with [^{14}C] and [^{3}H]adenine was in the presence of cycloheximide for 2 hr (10); cells were washed, starved in phosphate buffer for 1 hr at 30° and then subjected to mutagenesis by 25 µM EB for 2.5 hr in buffer at 30°. They were then suspended in complete media containing 5 µM unlabeled adenine and 2% glucose or 3% lactate, respectively, and were harvested at the times shown. The samples were then combined as indicated, the cells lysed and the mtDNA analyzed in CsCl gradients as described previously (10) (see also Fig. 5). The value for EBIC in gradient C represents an overestimate and is probably due to nuclear contamination since no peak was observed in the density region corresponding to mtDNA. The increase in the counts in the control gradients are probably due to DNA synthesis using RNA as a precursor; this effect is always observed with nuclear DNA, and usually much less pronounced with mtDNA.

i.e. the effect of UV is predominantly on mtDNA. It appeared of interest to determine the possible effect of this class of deficiencies on EB induced lesions. We were fortunate to obtain 2 such strains and N123, their parent wild type, from E. Moustacchi. The mode of inheritance of the *uvs* ρ markers in these strains has already been investigated by her: The first, *uvs* ρ5, exhibits a normal Mendelian pattern of segregation and probably represents a normal chromosomal mutation. The pattern of the second, *uvs* ρ72, is abnormal, and thus it may be due to a cytoplasmic (presumably mitochondrial) mutation. This strain also exhibits an enhanced frequency of spontaneous ρ^- mutations, and appears more susceptible than the wild type to mutagenesis by EB after prolonged starvation (45). We have subjected the 3 strains to an extensive analysis with respect to most of the parameters governing EB mutagenesis, described in earlier sections, and some of our results are presented in Table 4. They may be summarized as follows: 1) The absence (or modification) of a component of the mitochondrial UV repair system in *uvs* ρ5 protects against, while the analogous deficiency in *uvs* ρ72 exacerbates, the action of EB. This is true for both starved cells grown previously either under derepressed (glycerol) or repressed (glucose) conditions, and for cells still growing under either of these 2 conditions. This enhanced rate in ρ72 is accounted for to

TABLE 3

Requirements for metabolic effects

Expt	Carbon source	Time at 30° (min)	% Wild type colonies on: Expt C, Glc grown cells — Plated on glucose-TTZ	Expt C, Glc grown cells — Plated on lactate	Expt D, Lac grown cells — Plated on glucose-TTZ	Expt D, Lac grown cells — Plated on lactate
1	glucose	0	0.9	100	10.5	100
		60	0.7	84.6	4.6	100
		120	1.3	63	4.1	95
		240	6.1	11	5.9	24.3
1	+ CH	240	5.2	24	6.1	74.3
	+ erythro	240			4.4	41.1
2	lactate	0	2.2	100	10.5	100
		60	3.2	69	4.3	100
		120	2.2	67	6.1	100
		240	2.7	85	43[a]	100
2	+ CH	240	7.9	100	4.9	80
	+ erythro	240			17.1[b]	60

CH = cycloheximide; Glc = glucose; erythro = erythromycin; Lac = lactate.

[a] 60.9 at 300 min.

[b] 24.3 at 300 min.

In each experiment cells were grown to mid-exponential phase on glucose (C) or lactate (D), washed, starved in buffer for 1 hr at 30° and treated with 25 μM EB for 20 min (C) or 35 min (D). Cells were then filtered, washed and suspended in fresh 1% glucose or 3% lactate medium (with or without cycloheximide or erythromycin) and incubated at 30° for the times indicated. At intervals cells were diluted and placed on 1% glucose or 3% lactate plates for determination of % ρ^+ . Zero time is the beginning of the post-EB incubation. Concentrations of cycloheximide and erythromycin were 100 μg per ml and 2 mg per ml, respectively. In these experiments CAP post-treatment resulted in very rapid curing while erythromycin had no such effect.

a large extent by a reduction in the initial shoulder of the kinetic curve, an effect frequently explained in radiation biology in terms of the elimination of repair events (42,43). 2) Protection — or a slower rate of mutagenesis — in *uvs* ρ5 is not due to impermeability of its cells or mitochondria to EB, since it is as susceptible to inhibition of the synthesis of mtDNA as in the wild type. 3) In spite of their mutation-prone state, cells of *uvs* ρ72 still are capable of being affected by the various phenotypic manipulations described earlier: competition by Antimycin A (at 1 and 0.05 μg per ml), reversal by this agent at these concentrations, as well as by acridines and by heat treatment; metabolic reversal by lactate and to a lesser extent by glycerol. However, although qualitatively similar, quantitatively the agents or treatments are consistently less effective than in the wild type and generate a large proportion of cells giving rise to mixed (ρ^+ plus ρ^-) clones.

A detailed description and analysis of these results and of the properties of the *uvs* ρ strains in general is being assembled jointly by E. Moustacchi and ourselves and will be published elsewhere. For our purpose here the permissible

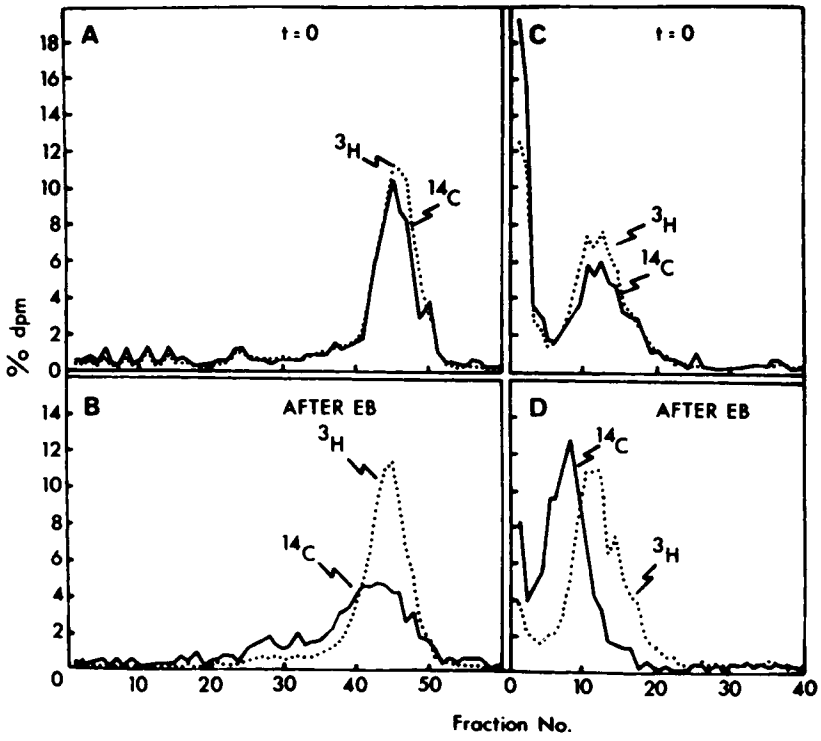

Fig. 5. EB-induced alterations in parental ρ^+ mtDNA. Cells of IL-8-8C, a haploid *his⁻try⁻* auxotroph grown on lactate medium to A_{600} = 0.8 were labeled with [^{14}C] (4 µCi per ml) or [^{3}H] (196 µCi per ml) adenine for 2 hr in the presence of cycloheximide (10). Cells were washed, an aliquot of each batch of cells saved for zero time analysis and the remainder starved for 1 hr at 30° in buffer. The [^{14}C]labeled cells were then treated with 25 µM EB for 2.5 hr so that the population was converted to <1% ρ^+. Appropriate [^{14}C] and [^{3}H]labeled samples were mixed and converted to spheroplasts; half of each sample was used for the usual CsCl analysis (panels A and B — sedimentation to the left) while the other half was lysed with sodium lauryl sarcosinate and sedimented into a 5 to 20% neutral sucrose gradient for 4 hr at 26,000 rpm at 15° using an SW-27 rotor and a Beckman Model L2 ultracentrifuge (panels C and D — sedimentation to the left). Total dpm of [^{14}C] recovered was 6480, 3870, 9360 and 4950 for gradients A-D, respectively, and dpm of [^{3}H] recovered was 1,568,000, 1,193,000, 1,247,000 and 855,200 for gradients A-D, respectively. The recovery of counts in the mtDNA regions (buoyant density = 1.685 g per ml) of all gradients was 100 ± 30%. The counts in fraction 1-4 of gradient C represent adenine-containing material which has not entered the gradient but which has a high enough molecular weight to be precipitated by TCA and caught on Whatman GF/C papers. These counts are probably due to a small fraction of RNA which has survived the base hydrolysis step, since in other studies it has been shown to be metabolically labile in contrast to authentic labeled DNA. In other studies we have demonstrated that nuclear DNA, when labeled, is found in a well-resolved (from mtDNA) peak around fractions 25-30 of sucrose gradients (*cf* panels C-D). In this particular case less than 5% of the total counts was in this component.

TABLE 4

Mutagenic parameters for uvs ρ *strains*

EB (μM)	Time of exposure (min)	Treatment nature	% ρ⁻ mutants in population		
			N 123	*uvs* ρ5	*uvs* ρ72
	(0 time)		0.4 ± 0.3	3.3 ± 0.3	24.5 ± 0.5
25	20		35.6	2.7	55.2
	30		82.4	7.3	94.5
	60		92.7	17.6	>99
	60	Plate on gly	47	0	75[a]
	60	Antimycin A[b]	1.4	3.3	39.5[c]
25	30		34.2		70
	60		72.8		89.7
	60	Antimycin A[d]	7.0		41.1
	60	Antimycin A[e]	21.8		60 (52.2)[h]
20	45		84[i]		90
	45	Acriflavin[f]	10.3[i]		31
	45	Heat[g]	17.0[i]		40

[a] 65% at 35 min. [b] 0.25 μg per ml during mutagenesis.

[c] 47% with 1 μg Anti A per ml during mutagenesis. [d] 0.05 μg per ml.

[e] 0.05 μg per ml for 90 min subsequent to mutagenesis.

[f] 100 μM for 90 min subsequent to mutagenesis.

[g] held at 45° for 90 min subsequent to mutagenesis.

[h] 30 min exposure to EB. [i] 120 min.

All experiments were done on cells grown on 3% glycerol medium to mid-exponential phase, harvested, suspended to the same turbidity for all 3 (or 2) strains tested, starved in buffer for 60 min and exposed to EB for the time indicated; the actual cell concentrations during mutagenesis varied between 2 to 5 x 10^6 per ml in the 3 experiments shown but was the same within any one experiment. Cells were plated on 1% glucose medium with one exception as noted.

inferences from these results would appear to be that: 1) A component of the mitochondrial (excision?) UV repair system, specified by a nuclear gene, fulfills an essential function in EB mutagenesis, perhaps in the recognition and modification of a complex between parental mtDNA and EB. 2) In the absence of a second component also required for effective repair of UV-induced lesions in mtDNA, but of mitochondrial specification, mutagenesis by EB is accelerated, perhaps by virtue of less efficient (recombination?) repair subsequent to the initial lesion described under 1). 3) When the component(s) described under 2) is rendered ineffective by mutation, the entities required for phenotypic protection and reversal by the particular agents described, specifically by heat treatment or binding of acriflavin and Antimycin A, is modified as well.

Fatty acid desaturase mutants. If attachment of mtDNA to, or interaction with, the mitochondrial inner membrane is important in EB mutagenesis as suggested by some of the phenotypic effects described, substantial modification of the former should exert some pronounced and measurable influence on the latter.

One of the best ways of manipulating membrane composition, and perhaps modify its function, is to modulate profoundly the nature of the unsaturated fatty acids in its phospholipids by growing a mutant auxotrophic for unsaturated fatty acids (46,47) on various defined supplements of the latter (24). Accordingly we have compared the *ole*-1-2 mutant strain kindly furnished us by A. Keith grown on minimal media containing 2 x 10^{-4} M of 2 structurally quite disparate, monounsaturated C_{18} fatty acids, i.e., oleic (*cis*-Δ^9-$C_{18:1}$, m.p. ≤5°) and petroselinic (*cis*-Δ^6-$C_{18:1}$, m.p. 28°) acid respectively. The growth curves for the 2 samples were identical, but they differed in their susceptibility to EB. Some of the parameters governing their EB mutagenesis under standard nongrowing conditions, with and without prior heat treatment are summarized in Table 5. We observe, as anticipated, enhancement of mutagenic efficiency as a result of the introduction of the inherently more destabilizing petroselinate into the phospholipids of the mitochondrial membrane. The actual rate is enhanced and a higher temperature is required for thermal protection.

These results, as well as the fact that for mutagenesis at 30° it is the cells grown on petroselinate — the component that might be suspected to form a more "solid", hence less easily penetrated, membrane — that are more susceptible to mutagenesis, probably rule out differential permeability to EB as a likely explanation.

TABLE 5

Influence of fatty acid composition of membranes on mutagenesis

Treatment		% ρ^- colonies with cells grown previously on: Oleate	Petroselinate
Zero time		0.15	0.15
Mutagenized control		13.1	22.0
Mutagenized after treatment at			
42°	30 min	6.9	18.0
42°	60 min	4.7	20.9
45°	30 min	5.1	11.1
45°	60 min	5.2	9.1

Cells of strain KD115 were grown on minimal medium with 3% lactate as carbon source and supplemented with fatty acids at 2 x 10^{-4} M. They were harvested at a cell density of <10^7 cells per ml, centrifuged, washed and starved as described for previous experiments. Heat treatment was similar to that described for Table 1 at the temperatures and for the periods shown. The controls were kept at 30°. All samples were mutagenized with 25.0 μM EB at a cell density of 4 x 10^6 per ml, and aliquots were withdrawn for plating on glucose-TTZ every 10 min. The values in the table represent the averages for the 50 and 60 min time periods (4 plates total). Zero time values represent the average for the control and various heat treated cells since heating appeared to have no effect on survival or extent of spontaneous mutagenesis.

4. DISCUSSION

The most puzzling features of the inhibitory and mutagenic effects of EB (as emphasized by several investigators) have always been its great selectivity for mtDNA, and its distinctiveness from other intercalating dyes, such as acriflavin, in producing mutagenesis in the absence of growth. These 2 observations are hard to rationalize in terms of any model that envisages strong intercalative binding of EB as a necessary and sufficient first step in the sequence of mutagenic events. Neither the difference in base composition nor the presumed circularity of mtDNA can really serve as an attractive physical basis for a plausible model. The former, if it influences EB binding at all does so only weakly (2,48-50), and is absent, in any event, in the case of DNAs from many vertebrates (51,52). The latter may not be an important determinant in the mtDNA from *Ascomycetes* (53), and in addition some circular DNA molecules are resistant to the action of EB (3,54), even in yeast (55). Furthermore the postulate of intercalation as a necessary event in ρ^- mutagenesis in general is eliminated by our findings, to be published elsewhere, that Berenil (56), an agent incapable of intercalation on theoretical grounds and operationally ineffective in this capacity with circular DNA (1), is nevertheless an effective mutagen reminiscent of EB in many of its properties. Berenil has also been reported to interact specifically with the DNA of trypanosomal kinetoplasts (56)

Intercalation even as a sufficient prerequisite is rendered unlikely by many of the observations reported here. In particular, there is no evidence that Antimycin A at a level of 0.25 µg per ml or less is capable of forming a stable complex with any DNA thus rendering it resistant to complexation with EB. Similarly heating DNA to 45° in an intracellular environment of relatively high ionic strength would not be expected to lead to such a profound modification, although some subtle alteration, such as a single bond sciscion in a particularly susceptible region of a supertwisted covalently circular mtDNA, cannot be ruled out at this time. Incidentally, any postulated interaction of EB with a mitochondrial RNA (56) rather than DNA would suffer from similar deficiencies. Other models, such as mutagenesis by the gradual and progressive accumulation of replication errors (57), perhaps brought about by the DNA polymerase which has been investigated *in vitro* (7), also no longer appear tenable. It only takes 3 cell generations or less before a mutant population becomes irreversibly established; and the hypothesis provides no ready explanation for the resistance of certain mutant strains, particularly of *uvs* ρ5, to the deleterious action of EB, nor can it account for the difference between KD115 grown on oleic or petroselinic acids.

What constitutes a feasible alternative to intercalative binding of EB to DNA? One likely target consistent with some of the effects described, including the resistance by ρ^- cells and the phenotypic effects of fatty acids may be provided by the mitochondrial inner membrane. The studies of Gitler *et al.* (59) clearly indicate that EB can interact with such membranes. Its fluorescence becomes enhanced as a consequence of such interactions, and reduced when they are modulated by anaerobiosis or interaction with Antimycin A. The membrane is also implicated as a target in a related context by the observation that Mikamycin, an inhibitor of mitochondrial protein synthesis, is also a respiratory inhibitor acting between cytochromes b and c_1, i.e., in the region sensitive to Antimycin (60). This fact has been made the basis of a "unitary hypothesis" causally linking the 2 phenomena (33,60). There also appears such a link between membrane configuration and formation of the mt ribosomal RNA in lipid depleted, anaerobic cells (33,61). Furthermore, the effects of substituting different fatty acids on membrane configurations are well known (e.g. 62,63) and provided the conceptual framework for the experiments testing their effect on mutagenesis.

However, interaction of EB with this membrane as a sufficient requirement can also be ruled out. While such an interaction accounts for mitochondrial relative to nuclear selectivity, it does not explain the specificity of EB relative to acridines which show completely analogous membrane binding and

fluorescence behavior (57), even in the case of compounds known not to be strongly mutagenic (20,21,35). Also, examination of sucrose and CsCl gradients (10) (Table 2 and Fig. 5) suggests that mtDNA undergoes some modification and partial degradation as a result of the initial exposure of starved cells to EB. Finally, although the *uvs* ρ mutations may well involve or modulate components of the mitochondrial inner membrane, there is some reason to doubt that the incision-defective mutation in *uvs* ρ5 has produced a structural alteration exclusively in the inner membrane, at a site removed from mtDNA.

All the observations described are, however, completely consistent with a model that postulates a complex between mtDNA and the inner membrane as the initial site of interaction with EB (24). This interaction then generates a distortion of the DNA within the complex which prevents its replication and renders it recognizable, and particularly susceptible to attack by the (so far largely hypothetical, but see 41,44) intramitochondrial system(s) capable of repairing damaged mtDNA (for details of the procaryotic equivalent see 42,43, 65,66). The intimate association with, and attachment of mtDNA to, the inner membrane is reasonable on the basis of what is known about the requirements for the replication and repair of the DNA of procaryotic cells, including their plasmids (54,65-69), and of morphological evidence already available (70). The distortion of such a complex and its resultant ready susceptibility to modification and degradation as a consequence of changes induced in its membrane attachment site is based on the evidence presented here and is subject to ready tests by physical and chemical means. As a corollary, also shown here, chemical or biological effects on mtDNA, and their consequences, may provide investigators with a novel, simple, and convenient probe of membrane function.

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