A NOVEL REACTION OF MITOCHONDRIAL DNA WITH ETHIDIUM BROMIDE

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1. Introduction

Probably the most spectacular action of the drug ethidium bromide (Etd Br) is its quantitative conversion of cells of *Saccharomyces cerevisiae* – a facultative anaerobe – into respiration-deficient mutants, caused by a permanent and heritable alteration in the mitochondrial genophore of this organism. Although first observed in 1968 by Slonimski and his collaborators [1], the primary cellular events induced by Etd Br that produce this dramatic effect are by no means completely understood. Among the phenomena reported to result from treatment of yeast cells by Etd Br are severe alterations in the structure of the inner mitochondrial membrane [2, 3], inhibition of synthesis of cytochromes $a + a_3$, b and c_1 in that order [2–4], inhibition of the synthesis of mitochondrial (mt) RNA [5, 6] and a progressive fragmentation of mtDNA leading ultimately to its complete destruction [7–9].

Although Etd Br has been known for some time to be capable of intercalation – i.e. of insertion between adjacent base pairs in the DNA helix, leading to distortions in its structure [10–13] – a simple intercalative model is probably not sufficient to account for the variety of effects reported. This applies in particular to its specificity with regard to the nature of the cells and DNAs affected and to the conditions that distinguish the action of Etd Br from that of other intercalating dyes [14].

So far there have been few attempts at establishing the mechanism of action of Etd Br at the molecular level and none that provide a satisfactory explanation of its specificity. A powerful tool for this purpose is the ready availability of radioactively-labelled Etd Br, recently synthesized in this laboratory [15]. The results here presented are the first fruits of its use. They show that at least one step in its action involves the formation of a TCA-precipitable product of Etd Br associated uniquely with mtDNA, suggesting that a covalent bond may be formed between these two moieties early during the process leading to mutagenesis.

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2. Materials and methods

2.1. *Materials*

All chemicals are reagent grade; calf thymus DNA was purchased from Sigma Chemical Co.; [^{14}C]adenine (specific activity 55 mCi/mmole) was purchased from New England Nuclear; Hyflo Super-Cel was from Fisher Chemicals, while poly-lysine was from Sigma Chemical Co.; Glusulase (a crude enzyme from snail gut juice) was from Endo Labs; [^{3}H]Etd Br (specific activity 3 mCi/mmole) was synthesized according to the procedure of Bastos and Mahler [15].

2.2. *Cell lines and growth conditions*

A commercial strain of *Saccharomyces cerevisiae* (Fleischmann), and two haploid strains – IL-16 and IL-8-8C/R2, mitochondrial genotypes respectively $\rho^{+}C^{S}E^{S}$ and $\rho^{-}C^{0}E^{0}$ – both gifts from Professor P. Slonimski, were used throughout this work.

All cells were grown in a medium (RHT) containing 0.2% yeast extract (Difco), 0.1% $(NH_4)_2SO_4$, 0.1% KH_2PO_4, 0.5 $MgSO_4 \cdot 7H_2O$, 0.5 NaCl, 0.02% histidine and 0.02% tryptophan, supplemented with

either 2% glucose or 3% sodium lactate as carbon sources, as specified. The cells were initially taken from a slant and grown to stationary phase in RHT + 2% glucose. For the actual experiments we used an aliquot of this culture, inoculated it into fresh sterile medium at a 1:1000 dilution, and grew the cells with aeration at 30°C to the desired concentration (measured by turbidity at 600 nm, A_{600}) determined in a Zeiss spectrophotometer.

2.3. *Kinetics of mutagenesis*

Cells were grown under the desired conditions and starved in PO_4 buffer (pH 6.5) for 1 hr before addition of the drug. Etd Br was then added in the desired concentration, samples taken periodically and, after appropriate dilution, plated in RHT + 0.1% glucose + 3% glycerol + 2% agar, a medium in which after 2–3 days of growth at 30°C respiratory-sufficient cells give rise to large colonies, while respiratory-deficient mutants produce pinpoint colonies.

2.4. *Isolation of mitochondria*

Cells were harvested at 600 *g* at the desired A_{600} and the wet weight determined. For each gram of cells, we added 1 ml of water, 0.5 ml of a 0.1 M solution of Na_2EDTA and 0.5 ml of a solution of β-thioethylamine hydrochloride (60 mg/ml). The pH of the suspension was adjusted to 6.8–7.0 with 1 M NaOH. The cells were incubated at 30°C for 30 min, and then collected for 5 min at 5000 rpm at room temperature. The pellet was resuspended in 1 ml/g of cells of 1.8 M sorbitol + 0.34 ml of a citrate–phosphate buffer (obtained by titration of 1 M citric acid with 1 M KH_2PO_4 to pH 5.8). To this suspension, 1 ml of glusulase was added for each 5 g of cells. Incubation of the mixture at 30°C for 90 min converted > 90% of the cells into spheroplasts. These were harvested and gently resuspended in 1 M sorbitol, centrifuged, and this washing procedure repeated twice more to insure the removal of all the enzyme. This preparation constitutes the washed spheroplasts used in some of the experiments.

For isolation of mitochondria, the washed spheroplasts were suspended in 20 ml of 0.5 M sorbitol, 20 mM $MgCl_2$, 10 mM KCl and 5 mM KH_2PO_4 (pH 6.8) and blended for 15 sec at low speed followed by 15 sec at high speed. The homogenate was centrifuged at 2000 rpm (Sorvall RC2, SS-34 rotor) for 10 min, the pellet discarded and the supernatant recentrifuged at this speed until no visible pellet remained. After that, the supernatant solution was centrifuged at 13 000 rpm for 20 min to collect the crude mitochondria. The particles were resuspended in the same buffer as above, except that the $MgCl_2$ was substituted by 100 mM EDTA, and again spun at 13 000 rpm (Sorvall RC2, SS-34 rotor) for 20 min. The pellet constituted the washed mitochondria.

2.5. *Sedimentation analysis of DNA on sucrose gradients*

Either washed spheroplasts or washed mitochondria, as specified, were suspended in 1.8 ml of a saline buffer (0.15 M NaCl + 0.015 M trisodium citrate, pH 8.1) and mixed with 0.2 ml of a 10% solution of sodium lauryl sarcosinate (SLSa). The mixture was left standing at room temperature for about 15 min, after which it was loaded on the top of a 10–30% sucrose gradient (containing 0.005 M Tris, 0.005 M NaCl and 0.01 M EDTA, pH 8.1). The gradient was centrifuged in a SW 27 rotor of a Beckman ultracentrifuge at 18 000 rpm for 19 hr at 5°C. After this, it was fractionated in 1 ml fractions extruded from the top by introducing 60% sucrose solution into the bottom of the tube.

The fractions were incubated overnight at room temperature after addition of 0.2 ml of 2 N NaOH. They were neutralized with 0.56 ml of 3 M NaH_2PO_4 and immediately precipitated with 0.44 ml of 50% TCA. They were stored at 4°C overnight and filtered through glass fiber filters (GF/C Whatman) which were subsequently rinsed with 10 ml of 10% TCA and 10 ml of ethanol. The dried filter papers were counted in a Tri-Carb Packard liquid scintillation counter in scintillation fluid (32.5 g of PPO and 1.95 9 of POPOP in 6 litres toluene). Spillover of counts among the various channels of the counter were corrected by standard curves. In control experiments (> 10) mitochondrial (mt) DNA bands in a broad peak centered on fraction 16, which is shifted upon incubation of cells with Etd Br to yield a predominant peak around fraction 10 [16]. Labeled T7 DNA (s = 32.0 S) added as an internal standard exhibits a narrow peak also in fraction 16.

2.6. *Density analysis of DNA on CsCl gradients*

Either washed spheroplasts or washed mitochon-

dria, as specified, were suspended in 0.8 ml of 0.02 M Tris HCl (pH 8.5) and the sample was pipetted into a tube containing 0.2 ml of 10% SLSa, shaken well to homogenize and left standing at room temperature for 10 min. To the homogenate were added 3.36 ml of a CsCl solution (26 g of CsCl in 14 ml of 0.02 M Tris HCl, pH 8.5) and the refractive index of the resulting solution adjusted to 1.3980 at 30° C. The remainder of the tube was filled with mineral oil and sedimentation to equilibrium obtained by spinning the sample at 33 000 rpm in a Type 40 rotor of a Beckman ultracentrifuge at 20°C for 60–72 hr.

The gradient was fractionated in 0.1 ml fractions; to each of them were added 20 μl of 1 N NaOH and the samples incubated overnight at room temperature. After that, we added 10 μl of 1 M NaH_2PO_4 and the whole sample was plated on GF/C Whatman filter papers and allowed to dry. The dried filters were washed 3 times, 10 min each, with cold 5% TCA, once with a 3:1 ethanol/ether mixture and once with ether alone, dried and counted in scintillation fluid as above.

2.6. *Polylysine-Kieselguhr (PLK) chromatography*

The procedure utilized for preparation of the sample and construction of the column were as described by Finkelstein et al. [17]. The column was eluted with a total volume of 100 ml of a linear gradient from 1.7 to 2.3 M NaCl solution buffered with 0.02 M KH_2PO_4 at pH 6.8. DNA was monitored by reading A_{260} of the 1 ml fractions. For measurement of DNA-associated, TCA-precipitable Etd Br, the fractions were treated in the same way as described above for the sucrose gradient fractions.

2.7. *Thin layer chromatography for separation of nucleotides*

DEAE-cellulose (Cellex-D) was suspended in water in a proportion of 15 g/litre. Five g/litre of $CaSO_4$ was added to promote adherence to the plate. The suspension was applied to 20 × 20 cm glass plates (100 ml/plate) and permitted to dry at room temperature for 24 hr.

Development of chromatograms was bidimensionally utilizing the system proposed by Randerath and Randerath [18]. Development i the first dimension was by 0.25 M LiCl, in the second by 1.3% H_3BO_3 in 0.33 M LiCl, with an intermediate drying of the plate. The dried plates were read by spotting the fluorescence of the compounds examined under ultraviolet light. Various nucleotides, Etd Br and all possible combinations of these compounds were used as standards.

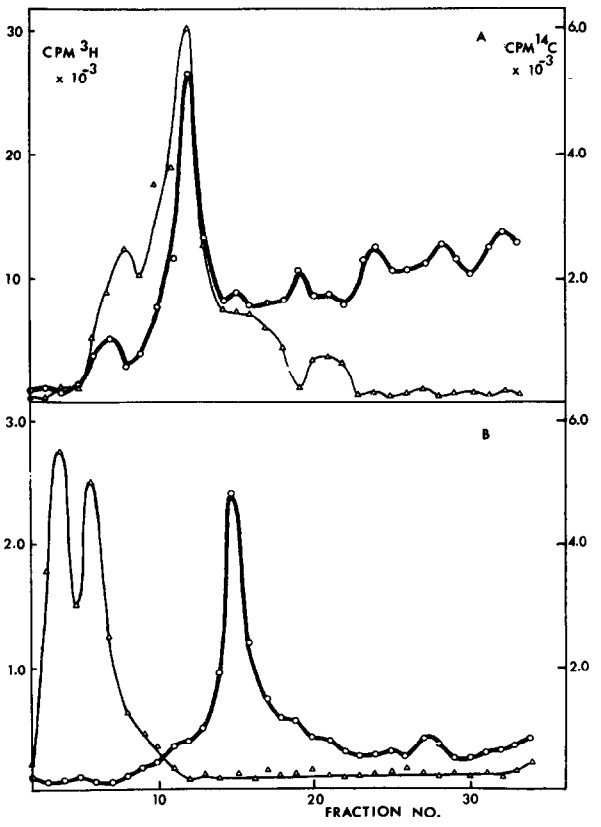

Fig. 1. Sucrose gradient analysis of mitochondrial DNA resulting from (A) in vivo incubation of [^{3}H] Etd Br with starved Fleischmann cells and (B) in vitro incubation of [^{3}H] Etd Br with a mitochondrial lysate from the same cells. (Counts on top of gradient are probably due to Etd Br occluded by membranes.) (o–o–o) cpm ^{14}C (DNA); (Δ–Δ–Δ) cpm ^{3}H (Etd Br).

2.8. *Other methods*

Paper electrophoresis was at 500 V for 7 hr, utilizing a Tris buffer, pH 8.0 as electrolyte. *Fluorescence spectra* were determined in a Aminco fluorimeter; excitation and emission wavelengths were as indicated; *absorption spectra* were run point by point in a Zeiss spectrophotometer.

3. Results and discussion

3.1. *Isolation of TCA-precipitable ethidium bromide associated with mtDNA*

Yeast cells (Fleischmann) were grown in 200 ml of RHT + 3% lactate + 50 μCi [^{14}C] adenine to A_{600} = 1.0, when the cells were harvested and resuspended in phosphate buffer (0.1 M KH_2PO_4, pH 6.5) to the same cell density and starved with aeration for 1 hr. After this, they were divided into two flasks. To the first, but not to the second 3.3 μmoles containing 10 μCi of [^{3}H] Etd Br was added and incubation of both was continued for 2 hr at 30°C. Mitochondria were isolated, and after lysis with SLSa, the samples were sedimented through a sucrose gradient with the addition of 10 μCi [^{3}H] Etd Br to the second (control) sample immediately before loading on the gradient. The results of the analysis are shown in fig. 1 from which two inferences emerge:

(i) TCA-precipitable Etd Br is found associated with mtDNA when starved cells are treated with the drug.

(ii) Treatment of a mitochondrial lysate with Etd Br in vitro does not lead any significant formation of TCA-precipitable material (the counts in the control are below 10% of those in the experimental sample).

The experiment was repeated using the haploid IL-16 instead of the commercial strain and its DNAs subjected to analysis by both sucrose and CsCl gradients. We found that the pattern in the sucrose gradient exactly matched that previously shown for the commercial strain (fig. 1), while the CsCl gradient showed that TCA-precipitable Etd Br is uniquely associated with mtDNA; in spite of the fact that a much larger amount of nuclear (n)DNA is present in both gradients, none of the label is found bound to this species.

This high degree of specificity was confirmed by means of PLK chromatography. Cells of IL-16, grown without any previous labelling of the DNA and starved for 1 hr in phosphate buffer, were incubated with 10 μCi [^{3}H] Etd Br for 2 hr. The DNA was prepared, analyzed on columns as described in Materials and methods, and its profile is shown in fig. 2a. Again, TCA-precipitable Etd Br is found only with the mtDNA peak. Furthermore, if crude mtDNA is first isolated from Etd Br-free cells by a preliminary PLK chromatography and *then* incubated in vitro with

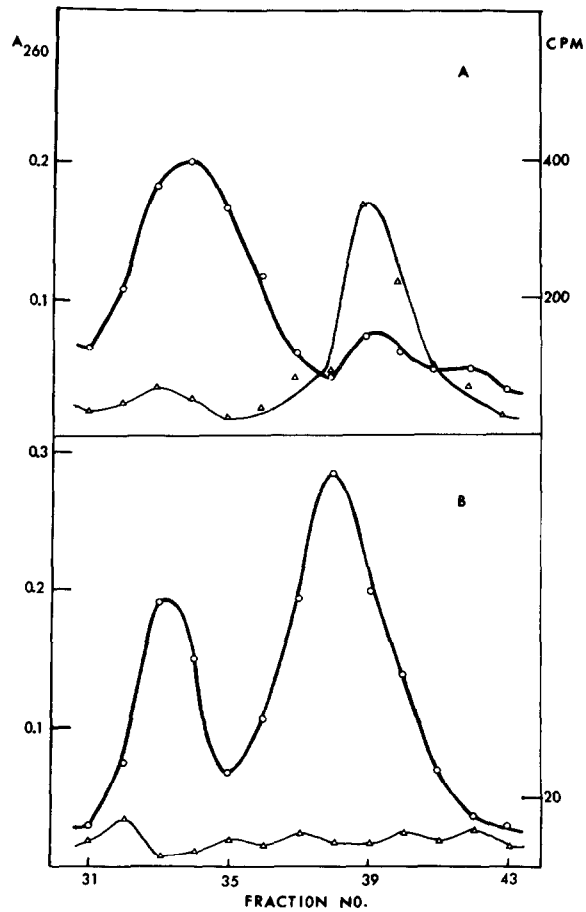

Fig. 2. PLK chromatography of (A) cellular DNA from IL-16 incubated with [^{3}H] Etd Br and (B) crude mtDNA isolated from Etd Br-free cells, subsequently incubated in vitro with [^{3}H] Etd Br. The first peak (fraction 33–34) corresponds to nDNA, while the second (fraction 37–38) corresponds to mtDNA. (o–o–o) A_{260} (DNA); (△–△–△) cpm ^{3}H (Etd Br).

10 μCi of [^{3}H] Etd Br, followed by chromatography of this sample, one obtains a very good resolution of nuclear and mitochondrial peaks, none of which contains any TCA-precipitable Etd Br (fig. 2b). The formation of this product therefore again depends on events taking place in vivo.

3.2. *Time course of incorporation*

Cells of IL-16, previously grown to early stationary phase in RHT + 3% lactate, were suspended (1 litre) and starved for 1 hr in phosphate buffer (0.1 M

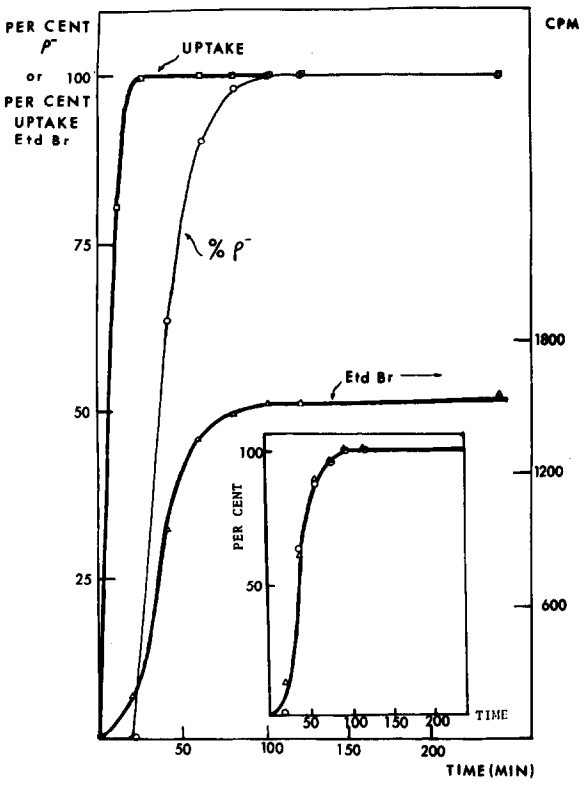

Fig. 3. Kinetics of mutagenesis and of formation of TCA-precipitable Etd Br: main plot shows kinetics of conversion of cells to respiratory-deficient mutants (ρ^-) and amount of Etd Br in cells and in a TCA-precipitable form. Inset shows results plotted as per cent of final value. Radioactivity at zero time was < 130 cpm. Uptake of labeled Etd Br was measured by filtering 5 ml of cell suspensions through Millipore filters, washing the latter extensively with water and ethanol and counting the dried filters in scintillation fluid. The plateau value found corresponds to about 9× that of the TCA-precipitable.

KH_2PO_4, pH 6.5) at an A_{600} = 0.5. 100 μCi of [^{3}H] Etd Br was added to the cells and 100 ml samples taken at the indicated times. TCA-precipitable Etd Br was estimated by the following method: the cells were harvested at 4°C and, after removing all remaining Etd Br by two successive phosphate washes, were incubated for 30 min in a β-thioethylamine hydrochloride solution (15 mg/ml) containing 0.25 M EDTA, broken with glass beads in a Braun shaker in a solution 0.4 M in NaCl buffered with 0.02 M KH_2PO_4 to pH = 6.8. The resulting homogenate was centrifuged at 27 000 *g* for 30 min, the supernatant incubated for 60 min at 30°C with heat-treated RNAase A (50 μg/ml), extracted twice with 10 ml isobutanol and precipitated with TCA (final concentration 10%). The precipitate was collected by filtering through glass fiber filters (GF/C Whatman) and counted in scintillation fluid.

The results are shown in fig. 3, together with the kinetics of uptake of Etd Br by the cells and their mutagenesis to respiration-deficient mutants. They indicate that except for a brief initial lag, formation of TCA-precipitable Etd Br (but *not* uptake) matches the mutagenic activity of the drug, suggesting a close link between the two processes.

3.3. *mtDNA^O petite – A biological control*

The obvious control to insure the specificity of formation of TCA-precipitable Etd Br with mtDNA is to repeat the experiment with a cell that lacks all mtDNA. One such strain is IL-8-8C/R2, a mtDNA^O petite [19]. Its cells were grown in RHT + 2% glucose to A_{600} = 1.0, starved for 1 hr and incubated with 10 μCi [^{3}H] Etd Br in buffer for 2 hr. During growth, 50 μCi of [^{14}C] adenine was present in the medium in order to label the cellular DNA. When lysed spheroplasts were centrifuged in a sucrose gradient, the pattern indicated a complete absence of TCA-precipitable Etd Br throughout the gradient including the prominent peak of nDNA. This result shows that the presence of mtDNA is required for the formation of the product.

3.4. *Other Controls*

Although the experiments described earlier had already indicated that interaction of Etd Br with DNA in vitro did not lead to any significant amount of TCA-precipitable drug, other controls were run to insure that the conditions utilized did not produce any artifact related to complexation of Etd Br with mtDNA. These are summarized in table 1.

Table 1
In vitro interactions of Etd Br and DNA.

Sample	cpm in sample	Fraction recovered
[^{3}H] Etd Br added	4×10^6	(1.00)
A	197	5×10^{-5}
B	< 5	$< 1 \times 10^{-6}$
C	< 5	$< 1 \times 10^{-6}$

Calf thymus DNA (30 A_{260} units) was dissolved in saline buffer and 10 μCi of [^{3}H] Etd Br was added to a final volume of 6 ml. This sample was divided into three aliquots. The first (A) was precipitated overnight at 4°C with 10% TCA, filtered through a Millipore filter and washed once with TCA and once with ethanol. The second (B) was loaded on a 5 X 0.8 cm Bio-Rex 70 ion exchange resin column, (Na^+ form) – a method long known to remove all intercalated Etd Br [20]. The column was eluted with 1.0 M KCl and the DNA-containing fractions precipitated, filtered and washed as above. The third fraction (C) was incubated overnight with 0.1 N NaOH at room temperature, precipitated, filtered and washed as above (simulating the treatment each of the gradient fractions receives before counting). Clearly, the various treatments described are able to eliminate completely any intercalated Etd Br from the TCA precipitable material.

The possibility that the TCA-precipitable radioactivity associated with mtDNA was actually due to a contaminant present in the [^{3}H] Etd Br preparation rather than labeled Etd Br itself was excluded by the following experiment. Cells were incubated under standard conditions with a constant amount of the labeled preparation but in the presence of variable amounts of purified unlabeled Etd Br. If formation of the product was due to a contaminant the total extent of incorporation should be independent of the unlabeled carrier, if it was due to Etd Br it should be diluted in proportion to the amounts of carrier added. The results obtained were completely consistent with the latter hypothesis.

3.5. *Characterization of TCA-precipitable Etd Br*

All the results presented so far are consistent with the possibility that mtDNA from starved cells treated with the drug contained a covalently bound form of Etd Br. We therefore isolated the fractions (total of 10 ml) containing this hypothetical complex in IL-16 from a sucrose gradient, utilizing lactate-grown cells labeled with [^{3}H] Etd Br under the standard conditions previously described. A 100 μl aliquot of this material was counted directly in Aquasol, giving a value of 39 600 cpm for the whole sample. It was treated overnight at room temperature with 0.1 N NaOH, dialyzed against buffered saline for 24 hr at 4°C and concentrated to a final volume of 0.5 ml by evaporation in vacuo. A 50 μl aliquot was again counted in Aquasol, giving 903 cpm for the radioactivity in the total sample. Thus of the Etd Br associated with the mtDNA peak approx 2.3% is in a form that can be precipitated with TCA. This value corresponds to one molecule of Etd Br for each 200 nucleotides. The sample was then lyophylized and redissolved in 0.5 ml Tris–Mg buffer (0.02 M Tris HCl + 0.02 M $MgSO_4$, pH 7.0), 50 μg DNAase I was added and incubated for 90 min at 37°C followed by 25 μg venom phosphodiesterase and the sample maintained for an additional 90 min at 37°C.

An aliquot of the solution containing the mtDNA digest was then applied to a DEAE-cellulose thin layer plate, developed in two dimensions and examined under ultraviolet light. It displayed four characteristic spots corresponding to the four main nucleotides of DNA plus an additional spot, with a very strong yellow–orange fluorescence close to the origin, which did not correspond to any of the standard nucleotides or to Etd Br itself.

We extracted the material from this spot with 0.1 N HCl and found it to account for all the Etd Br originally present in the digest of mtDNA. To check whether the Etd Br moiety in this compound could exchange with free Etd Br, the extract was incubated for 1 hr at room temperature with nonradioactive Etd Br (final concentration 10 μM); when this mixture was again applied to the plate and developed as before, the unlabeled Etd Br migrated to the position previously assigned to it, while the labeled, Etd Br-containing material was again left behind near the origin.

The physical properties of this material were examined to establish the differences between it and both free and intercalated Etd Br and summarized in table 2. Its properties appear distinct from the other two Etd Br species, shwoing that we are indeed dealing with a modified molecule. The structure of this compound is currently under investigation.

4. Conclusions

An analysis of the various observations taken in conjunction suggest the possibility of the existence of a covalent bond between Etd Br (or a derivative formed from it in vivo) and mtDNA. The formation

Table 2
Comparison of some physical properties of Etd Br, DNA-intercalated Etd Br (Etd Br_{DNA}) and Etd Br-containing spot from mtDNA digest (Etd Br_1).

Species	Maximum (nm)	Fluorescence* (Arbitrary units)
Fluorescence emission (excitation at 320 nm)		
Etd Br	585	0.06
	632 (shoulder)	0.02
Etd Br_{DNA}	585	0.8
Etd Br_1	515	0.8
	634	0.3
Fluorescence excitation (for strongest emission band)		
Etd Br	322	0.05
Etd Br_{DNA}	320	0.8
Etd Br_1	322	0.5
Visible light absorption		Extinction coefficient* (cm^{-1} M^{-1})
Etd Br	480	5.8×10^3
Etd Br_{DNA}	520	5.7×10^3
Etd Br_1	525	2.2×10^4
	Mobility (cm)	Net charge
Low voltage electrophoresis		
Etd Br	1.8	+
Etd Br_1	5.9	–
ATP	4.4	–

*Concentrations based on specific radioactivity.

of such a bond is not completely unexpected, since it has been reported to arise in tRNA under mild conditions, even in vitro [21]. The formation of a similar derivative by enzymatic or other action inside the mitochondria could account for the uniqueness of Etd Br in its mutagenic action toward *S. cerevisiae* and throw some light on the steps involved in this process.

We have presented here a correlation between the time course of formation of the TCA-precipitable Etd Br derivative and of mutagenesis; other studies (Bastos and Mahler, unpublished results) show a very good correlation between the amount of this material observed in various cell lines and their relative sensitivity to Etd Br. For instance, very little of it is found in petite mutants containing genetically meaningful mtDNA, cells known to be resistant to further degradation induced by the drug [19, 22, 23]; similar low reactivity is also exhibited by obligate aerobes in which the action of the drug is known to be reversible [2, 24, 25].

A model based on these results might postulate a series of steps that eventually culminate in mutagenesis with the insertion of covalently bound Etd Br into mtDNA as an early, though not necessarily the first, step in the sequence. This type of bonding could cause an alteration or distortion in the structure of the DNA – of an as yet unknown nature – that would increase its susceptibility to attack by some specific or non-specific nuclease(s), leading to its degradation. The only justification for the presentation of this hypothesis at this early stage is that it makes definite predictions which can be pursued experimentally.

Acknowledgements

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