STRUCTURAL REQUIREMENTS FOR MITOCHONDRIAL MUTAGENESIS

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Ethidium bromide (3, 8-diamino-5-ethyl-6-phenylphenanthridinium bromide) and euflavine (3, 6-diamino-10-methylacridinium chloride) are superficially similar in structure and ability to intercalate into DNA. However, they exhibit qualitative differences in their ability to bring about a mitochondrial mutation ($\rho^+ \rightarrow \rho^-$) in Saccharomyces cerevisiae. This investigation tried to establish and compare the essential structural prerequisites in three series of planar, heterocyclic dyes: the phenanthridines (P series), the acridines (A series), and molecules with different heteroatoms related to acridines (X series). Compounds capable of bringing about the mutation in the complete absence of growth and energy sources are restricted to di-primary amines in the P series: quaternization of the ring nitrogen, and an aromatic side chain at C-6 also appear essential. Compounds in the A series are mutagenic only with growing cells; quaternization (C_1 through C_4) is essential. The 10-allyl derivative is unusual; it is highly effective even in buffer supplemented only with an energy source. The results are interpreted in terms of a model that requires interaction of the mutagen with the mitochondrial inner membrane as well as with its DNA.

The conversion of respiratory competent (ρ^+) to cytoplasmically inherited, respiration deficient (ρ^-) yeast strains can be brought about by a variety of chemical mutagens. The most effective of these are the heterocyclic aromatic dyes such as certain acridines (1–3), and the phenanthridinium derivative ethidium bromide (3, 8-diamino-5-ethyl-6-phenylphenanthridinium bromide; Etd Br). As pointed out by Slonimski, Perrodin, and Croft (5), and amply confirmed since (6, 7), mutagenesis by Etd Br appears to be characterized by several unusual features in addition to being highly effective, since virtually quantitative mutagenesis (> 99.8%) can be achieved by exposure of starved cells, apparently in the complete absence of growth. Subsequent investigations showed that cells treated under these conditions are blocked in their ability to form progeny mitochondrial DNA (mtDNA) and are disposed toward the rapid degradation of the parental mtDNA (8, 9) when cellular growth is allowed to resume (10). Mutagenesis by Etd Br, or rather its eventual expression or fixation, also appears to be strongly affected by the intracellular (11-15) and specifically the intramitochondrial (7, 16) milieu. Again these effects are perhaps exhibited most dramatically under conditions in which the primary target and its putative function appear dispensable for mitochondrial activity and the mutagen is ineffective on mtDNA as in the following examples: (1) the competition with or reversal (curing) by Antimycin A of glycolysing cells under conditions of strong catabolite repression.* (2) Similar effects have been observed with acridines (16) such as euflavine, under conditions

*We have defined (7) three modalities of interference: a) protection, b) competition, and c) cure, depending on the time of addition of the interfering agent relative to that of the mutagen; for a) the agent is added and subsequently removed, prior to the addition of mutagen; for b) the two are present concurrently, while the mutagen is removed prior to the cure for (c).

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where they are completely ineffective mutagenically, as is dicussed below. As part of these continuing studies on the mechanism of mutagenesis by Etd Br it seems appropriate to ask what, if any, are the structural features in this molecule that confer on it these unusual properties, particularly when contrasted to the closely related acridine molecule. Specifically we have explored a large number of acridines and phenanthridine derivatives to determine whether a) certain structural elements are indispensable for effective mutagenesis and b) appropriate substitution can cause acridines to behave like Etd Br in their mutagenic characteristics. Conversely, can appropriately substituted phenanthridines behave like acridines? The results obtained suggest that Etd Br contains unique structural features, shared only with certain closely related analogues. Although an almost equally effective mutagen does exist in the acridine series in 10-allyl proflavine, the latter exhibits characteristics that set it apart not only from other acridines but from Etd Br as well.

METHODS

All experimental details not described in the text, including the source and growth of the yeast strains used, have been described by us previously (7, 10, 22).

RESULTS AND DISCUSSION

Mutagenic Efficiency under Standard Conditions

Experimental design. Previous studies (1–4) had indicated that mutagenesis by most heterocyclic dyes occurred only under growing conditions, probably because it was restricted in its action to progeny buds rather than to the parental cell (17). We therefore decided first to screen and compare the efficiency of all compounds to be tested on cells that were grown previously to mid-exponential phase on 2% glucose, harvested, washed, resuspended at 2×10^6 cells/ml, and then permitted to resume growth on 1% glucose with the potential mutagen at 25 μM. These are the conditions under which the two reference compounds, euflavine (A1 in Scheme I) and Etd Br (P1 in Scheme I), require approximately 200 min and 10 min, respectively, to convert 50% of the total cell population to mutant (ρ^-) colonies (Fig. I). We call this parameter τ, even though the usual semi-logarithmic plot of $[\rho^+ = (1-\rho^-)]$ / [total] versus time of exposure (dose) (Fig. I) exhibits a lag, not necessarily related to the number of hits or targets (5, 6, 18), followed in the limit by a straight line. The parameter τ, however, is sufficiently responsive to changes in conditions to justify its use as a basis of comparison. Since it is difficult to determine τ with any accuracy at 25 μM Etd Br, we also show the kinetics of mutagenesis with 5 μM Etd Br, and have included this measurement with every experimental set for purposes of normalization; in ten such sets the mean τ was 35 min with a range of 28 to 45 min. Similarly, other compounds with $\tau < 10$ min were also retested at concentrations of 10 and 5 μM. The compounds used are shown in Scheme I, together with their source. They fall into three series, the phenanthridines (Series P), the acridines (Series A), and other condensed heterocyclics formally related to acridine (Series X). An inactive compound is defined as one that produces $< 10\%$ ρ^- in 240 min or is inactive in reversal (see below).

Phenanthridines (Series P). The structural requirements in this series appear to be quite restrictive. Quaternization of the ring N^5 by a short alkyl side chain appears essential as shown by the Me derivative (compoung P3, "Dimidium"), which is highly active and still shows measurable mutagenesis (τ = 50 min) at 5 μM (Fig. I). However, activity disappears when R is provided by H (in compound P2 or P7), or by the complex side chain of "Propidium" [compound P5, which forms stronger complexes with DNA in vitro than

does Etd Br (19)]. An aromatic substituent at R_2 also appears essential as shown by the activity of compound P6 ($\tau < 15$ min at 25 μM). At this concentration continued exposure (>3 hr) to the agent produces an increasing number of nonviable cells. Interestingly enough, this lethality appears to be restricted predominantly to the ρ^- cells in the population. These side effects are eliminated on lowering the concentration to 10 or 5 μM (τ = 40 and 55 min, respectively). Compound P10, the benzyl analog of Etd Br, is comparable to compound P6 in activity (τ = 30 min at 5 μM). Substitution of Et for Phe at R_2 leads to a compound (P9) of quite low activity (τ = 200 min), but surprisingly a simultaneous alteration of Et to Met at R_1 (compound P8) restores some activity (τ = 10 min at 25 μM, 100 min at 10 μM). On continued exposure at high concentration there is evidence for some preferential inhibition without extensive lethality during the growth of the ρ^- cells in the population.

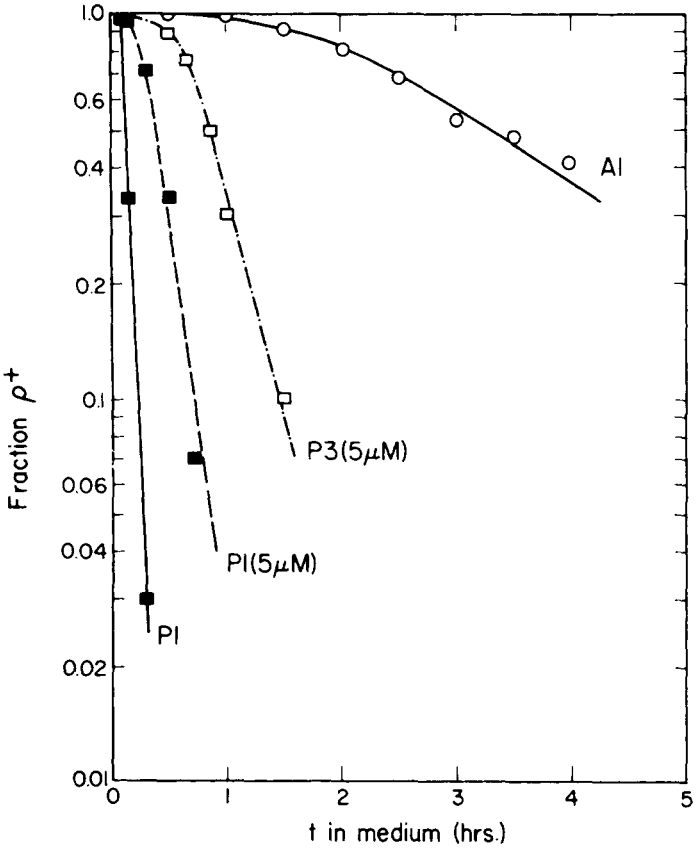


Fig. 1. Standard mutagenesis under growing conditions. S. cerevisiae (Strain Fleischmann) were grown overnight in 2% glucose medium (6) to mid-exponential phase ($\leq 10^7$ cells/ml, $A_{600} < 0.5$), harvested and washed. They were then resuspended (in foil-wrapped flasks) at a cell density of 2×10^6 cells/ml in fresh medium plus glucose, and the various mutagens at a concentration of 25 μM or as indicated (determined from their absorbance at their absorption maximum and published extinction coefficients) and then aerated vigorously at 30°C by shaking on a Gyratory Shaker. Generation time under these conditions was 75 min. Aliquots were removed and plated at appropriate dilutions for the determination of the proportion of ρ^+ and ρ^-cells in the population; the two methods commonly used, plating with glucose as the sole C-source, followed by tretazolium overlay (41) and plating on 0.1% glucose + 3% glycerol (5) gave identical results. The level of spontaneous ρ^- mutants in a population of these cells under these conditions is 0.8 ± 0.3%.

Acridines and related compounds (Series A and X). Previous studies (3, 4, 20) had already indicated the importance of alkyl substitution at the ring N (position R_1), the methyl derivative euflavine (compound A1) exhibiting much greater activity than proflavine, the H analogue (compound A2). We have confirmed these results and extended them to other alkyl derivatives, i.e., ethyl (compound A3), allyl (compound A4), and amyl (compound A5). The results at an acridine concentration of 10 μM are summarized in Fig. 2A. Compound A4 appeared the most active by far and was therefore selected for additional study (see below). We have also confirmed that in this series, as in Series P (3), only primary amines appear to exhibit any significant activity. Interesting also is the fact that the substitution of H by the highly hydrophobic t-butyl in positions 2 and 7 (compound A6) does not alleviate the inactivity of proflavine.

On the other hand, only tetramethyl phenosafranine (compound X2) and not the unsubstituted parent compound (X1) exhibits activity. X2 as well as X3 (pyronine) show activities comparable to that of euflavine (A1); τ at 25 μM is 180 min for X2, 160 min for X3, and 140 min for A1.

Other Mutagenic Parameters

Mutagenesis in the absence of growth. The compounds that were active with growing cells were re-examined for their effect on starved cells in buffer, conditions that are conducive to mutagenesis by Etd Br but not by euflavine. The phenanthridine derivatives found to be most active under growing conditions were also most effective with starved cells, i.e., P1, P3, and P6 at 25 μM all gave τ values $\leqslant$ 15 min. For P8, τ was 30 min but mutagenesis by this compound reached a maximum of only 70% ρ^- in 60 min; beyond this point the residual ρ^+ cells decreased somewhat in viability ($\sim$ 30% in 5 hr), while that of the mutants in the population was affected much more profoundly and was essentially completely destroyed within 5 hr. Compound P9 was virtually without activity (15% ρ^- cells in 6 hr). P1, P3, P6, and P8 were also re-tested at 10 and 5 μM. At the higher concentrations the τ values were 20 min for P1, 15 min for P3, 25 min for P6, and 10 min for P10, while P8 showed no effect; at the lower concentrations the τ values were 25 min for P1 and P3, 60 min for P6, and 80 min for P10. In contrast to these results in the P series, none of the compounds in the other two series exhibited any activity.

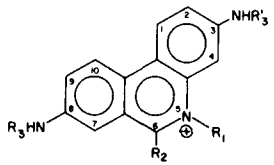
When these studies on acridines were extended to the use of starved cells in buffer in the presence of a C (and energy) source, we obtained the results shown in Fig. 2B. While other acridines were ineffective, allyl proflavine (compound A4) was highly effective at a concentration as low as 5 μM.

Cure of cells mutagenised by Etd Br. The inability of most of the acridine derivatives to be active mutagens in buffer afforded an opportunity to test their effectiveness in a related reaction, their ability to reverse or cure a previous mutagenic exposure to Etd Br (see footnote on p. 1). Euflavine had already been shown to be active in this regard (16) and we therefore extended such studies to all the compounds under investigation. The hope here was not only to derive some useful information concerning mechanism but also to rule out one possible cause for lack of activity, namely, a lack of penetration into the cells or their mitochondria.

The reactivity of various compounds may be compared by estimating, from data similar to those of Fig. 3, the length of time ($\bar{\tau}$) in buffer required to reverse effectively 0.5 of the cells subjected to a previous exposure to Etd Br with resultant mutagenesis to the 90% level. This order is for decreasing efficiency (increasing $\bar{\tau}$): i.e., ($\bar{\tau} \leqslant$ 10 min) A3

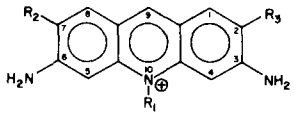
SCHEME I Structural Features of Potential Mutagens

Series P



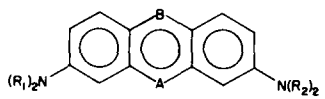
Compound	R_1	R_2	R_3, R_3'	Other	Source	Activity[a]
P1	Et	Phe	H	–	b	+
P2	H	Phe	H	–	c	–
P3	Me	Phe	H	–	d	+
P4	Et	Phe	Me	–	c	–
P5	$CH_2CH_2CH_2$ | +N (Me) Et	Phe	H	–	b	–
P6	Et	*p*-Me_2NH–styryl	H	–	d	+
P7	H	But	H	–	d	–
P8	Me	Et	H	–	d	±
P9	Et	Et	H	–	d	±
P10	Me	Benzyl	H	–	d	+
P21	H	Me	H	2, 7 diamino 3, 8, H	d	–

Series A



Compound	R_1	R_2	R_3	Source	Activity
A1	Me	H	H	b	+
A2	H	H	H	b	+
A3	Et	H	H	e	+
A4	Allyl	H	H	f	+
A5	Amyl	H	H	e	+
A7	H	t-But	t-But	f	+
A9	H	NH_2	H	f	+

Series X



	A	B	R_1, R_2		
X1	N–Phe	N	H	f	+
X2	N–Phe	N	Me	f	+
X3	O	C	–	f	+

[a] A plus means a τ or $\bar{\tau} < 240$ min, a minus, no detectable activity over this time. For definitions see text.

[b] Commercial, checked for homogeneity by chromatography and absorption spectrum.

[c] R. Bastos, of this group.

[d] Dr. T. I. Watkins, The Boots Company, Nottingham, Great Britain.

[e] Dr. G. Löber, Zentralinstitut für Mikrobiologie und Experimentelle Therapie, Jena, German Democratic Republic.

[f] Priv. Doz. Dr. W. Müller, Gesellschaft für Molekularbiologische Forschung, Stöckheim, Federal German Republic.

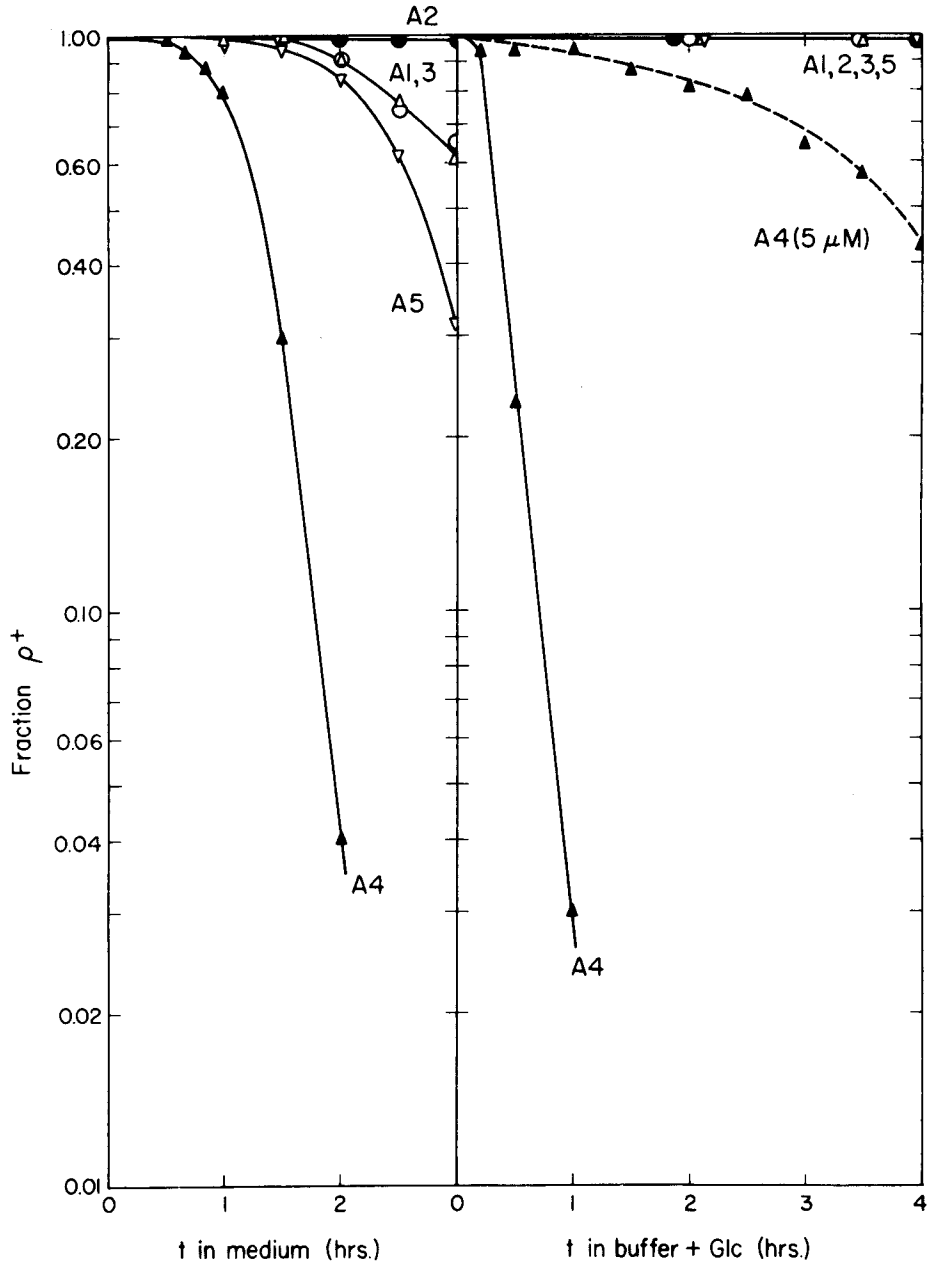


Fig. 2. A. Standard mutagenesis by acridines. The procedures followed were those described in the legend to Fig. 1, except that the concentration of mutagen was 10 μM unless otherwise indicated. B. Mutagenesis of starved cells. The procedure was as described in the legend to Fig. 1, except that after harvest and resuspension cells were first starved in 0.2 M phosphate buffer pH 6.5 with aeration for 60 min at 30°C. The cells were then divided into two equal portions. Mutagen at 10 μM (final conc) was added to both and glucose to a final concentration of 1% to one. The extent of mutagenesis at different times was again determined as before: no significant mutagenesis (<5% ρ^-) was obtained with any of the compounds tested for 4 hr in buffer alone.

$\geqslant$ A4* $\geqslant$ A5 $\geqslant$ A1 ($\bar{\tau}$ = 15 min) > A2 > A6 ($\bar{\tau}$ = 75 min); ($\bar{\tau} \simeq$ 10 min) A8 > X3 $\geqslant$ A1 $\geqslant$ X2 > X1 ($\bar{\tau}$ = 60 min) >> quinacrine. Evidently all the acridines with the exception of quinacrine are capable of entering the cell and mitochondria and of interfering with one of the entities or reactions required for the expression or consolidation of Etd Br mutagenesis.

The phenanthridines found to be ineffective by other criteria (P2, P4, P5, P7, P9) were found to be equally ineffective in reversal.

TABLE I. Characteristic Mutagenic Parameters

	Mutagenesis in			Interference by		
				Cure of	Competition with	
Mutagen	Medium	Buffer	Buffer + C	Etd Br	Etd Br	Anti A
Etd Br (P1)	+++[e]	+++	+++	+[a]		+
Euflavine (A1)[b]	+	–	–	++	++	±[d]
Allyl proflavine (A4)	++	–	++	+	+	–
Berenil[c]	++	–	++	+		±[d]

[a]On prolonged incubation of starved, repressed cells at a concentration $\geqslant$ 10 μM (R. Bastos, unpublished).

[b]Or any of its homologues.

[c]Mahler, H. R., and Perlman, P. S., Gen. Molec. Genetics, 121:285 (1973).

[d]% ρ^+ survivors increased 2 X.

[e]The number of plus signs indicates relative efficiency of mutagenesis (decreasing τ) or of interference (decreasing $\bar{\tau}$), as described in the text. A minus sign indicates absence of activity (τ or $\bar{\tau}$ > > 30 min).

Characteristics and Mechanisms of Mutagenesis

The discovery that N^{10}-allyl proflavine (compound A4) is a highly effective mutagen has permitted an explicit comparison of the characteristics of its mutagenic action with those previously established for Etd Br and the nonintercalating (21) trypanocidal dye Berenil (22) [di-(4-amidinophenyl)-triazine-(N-1, 3) diaceturate]. The result of this comparison is summarized in Table I. Most of the parameters have already been discussed. Those not yet covered are competition by Antimycin A (1 μg/ml) and competition with Etd Br (see footnote on p. 1). The former, when measured with glucose-repressed cells under conditions of optimal mutagenesis in buffer, or buffer plus C source, is virtually quantitative for 5 μM Etd Br for the first 60 min, but is only weakly effective (Berenil) or ineffective (A4) in buffer plus C source. Competition with Etd Br is defined as the ability of the compound in question (tested at 25 μM) to prevent mutagenesis by Etd Br (10 μM) in buffer. Competition is virtually quantitative for euflavine (compound A1) for

*This position cannot be determined with great accuracy; at the level used for all other compounds (25 μM) exposure to allyl proflavine appears to produce a synergistic, rather than an antagonistic, effect to the previous exposure to Etd Br; but A4 is antagonistic at lower concentrations (e.g., 10 μM) where $\bar{\tau}$ = 30 min.

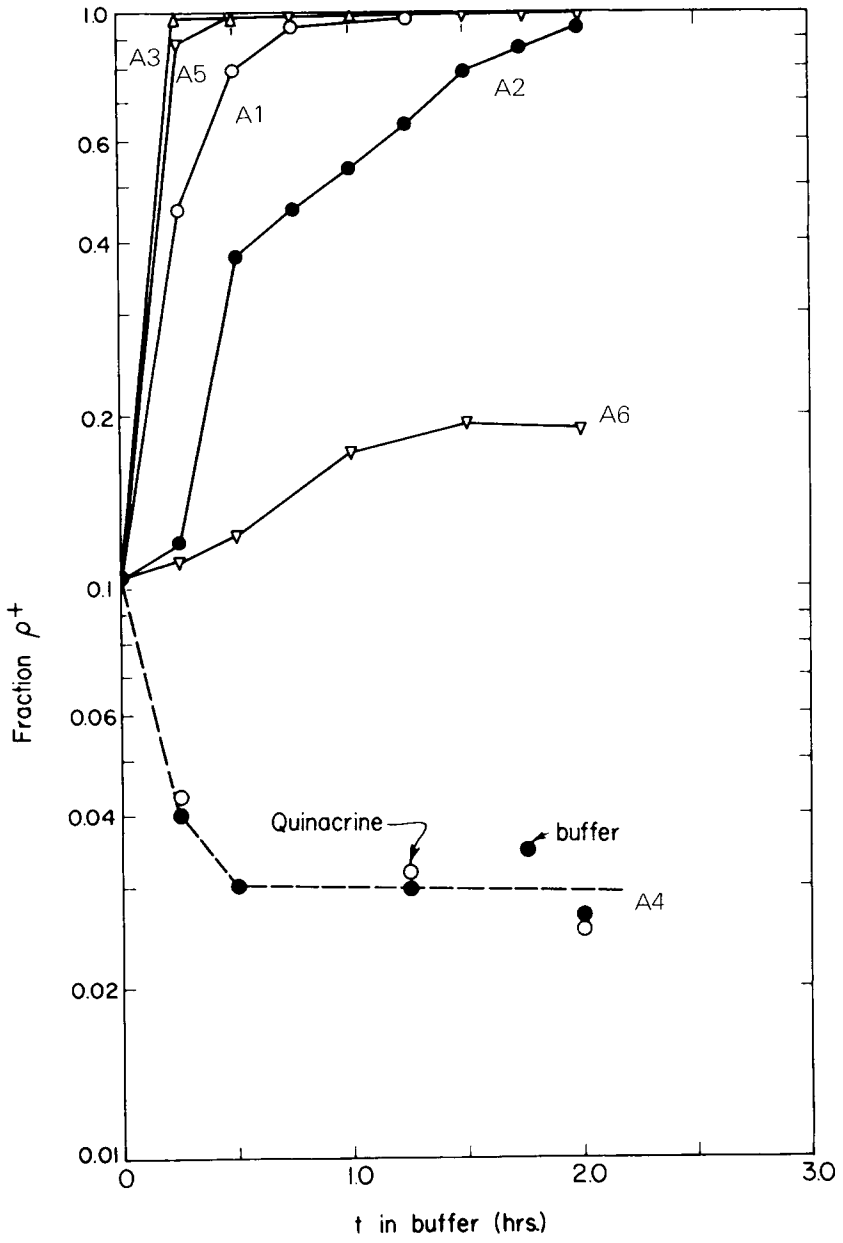


Fig. 3. Reversal (Cure) of Etd Br induced mutagenic events by other compounds. Cells, grown, pretreated, and starved as before, were first subjected to mutagenesis by Etd Br for 20 min in buffer (% ρ^- ~ 90%), freed of mutagen by filtration and washing, resuspended in 0.2 M phosphate buffer containing the compound to be tested (at 25 μM), incubated for the times shown, and assayed for mutants.

periods as long as 240 min, but only partially effective with compound A4 (55% ρ^+ survivors at this time).

It appears that mutagenesis by this agent obeys some of the rules previously established for Berenil rather than those characteristic of Etd Br or other N^{10}-alkyl acridines.

The basis for the high degree of specificity and selectivity of Etd Br (5, 11) (and certain closely related phenanthridinium derivatives) and for its detailed mode of action during the initial phases of mutagenic induction in buffer (5–7) is unknown. Intercalative binding to mtDNA as such is not a sufficient explanation, either from consideration of tightness of the interaction, or, if the DNA is in fact covalently circular, from consideration of its resultant under- or over-winding (19, 23–27). The affinity constants that can be expected to be operative in the intramitochondrial milieu for some of the compounds studied actually span only a relatively narrow range: e.g., at an ionic strength of 0.2, at 30°C, the extrapolated values at zero dye concentration can be estimated as 3,0; 0.5, 1.5, 1.3, 1.5; 2.5, and 1.5×10^5 M for Etd Br (P1) (23, 25); proflavine (A2), euflavine (A1), N-ethylproflavine (A3), N-amyl proflavine (A6) (28); N-allyl proflavine (A4) (29), and pyronine (X2) (30), respectively. These quantitative differences cannot be sufficient to explain the qualitative differences between the acridines (including N-allyl proflavine) and Etd Br, nor the two different patterns observed within the acridine series itself, or the qualitative difference between di-t-butyl proflavine and pyronine, where the order of binding constants is even in the wrong direction. Although there is a significant difference in winding angle (12° for Etd Br, 8–9° for the acridines studied) (21, 26, 27) this again is not sufficient to account for the qualitative differences in patterns between Etd Br and the alkyl proflavines, nor between allyl proflavine and the other alkyl derivatives. Furthermore, Berenil, which does produce a pattern similar to that of allyl proflavine, can neither intercalate nor affect the topology of covalently circular DNA (21). Finally, neither binding constants (28) nor winding angles (26, 27) for N,N′ alkyl substituted acridine derivatives appear grossly different from those of the primary amines, yet these are compounds that are ineffective mutagenically (3); similar considerations also apply in the phenanthridine series. Yet it appears likely that at least some of these compounds are capable of penetrating to and affecting the mutagenic site provided mutagenesis is initiated by Etd Br. It is interesting also that under conditions that accentuate the specificity of the initial mutagenic event, i.e., concentrations of mutagen $< 10\ \mu$M in buffer, activity is restricted to C-6 aryl substituted phenanthridinium derivatives.

A possible clue as to the origin of at least some of these differences – though not yet their underlying cause on the molecular level – is afforded by the observation that the first observable effect brought about by Etd Br in buffer is a modification of parental mtDNA, leading principally to a decrease in its size (and molecular weight distribution) but without any extensive formation of products of low molecular weight (7, 10). This initial (endonucleolytic) event, which may be called "registration," occurs either in the presence or the absence of a C source with Etd Br (Fig. 4A, B). However, it is strictly dependent on such an energy supply with mutagens such as Berenil (unpublished observations) – and probably allyl proflavine – while it is completely absent under either condition with euflavine (Fig. 4C). One may, of course, hypothesize that this difference may involve recognition of the complex between mtDNA and a given mutagen by two different, highly specific, sequence or conformation-recognizing nucleases which perhaps form part of the mitochondrial system for damage repair (33) – one nuclease being independent (Etd Br) and the other being dependent on a simultaneous requirement for ATP

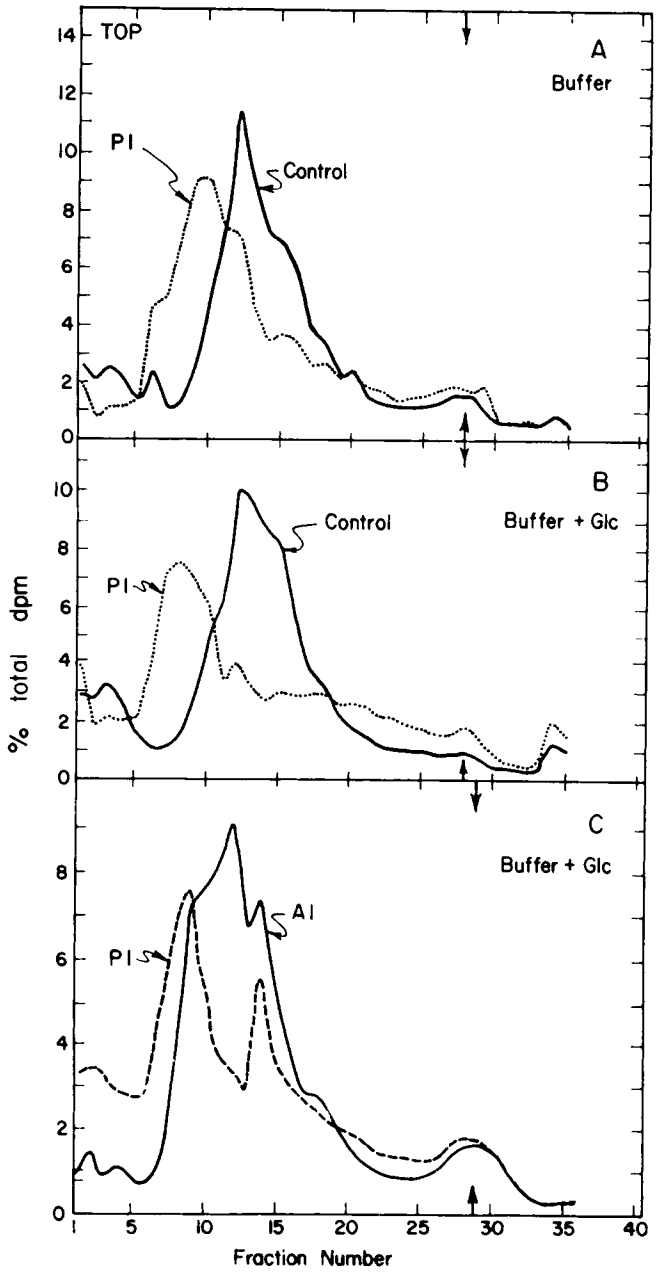


Fig. 4. A and B. The "registration" event induced in mtDNA by Etd Br. Cells of IL-8-8C (a His, Try auxotroph obtained from P. P. Slonimski) were grown on a semisynthetic medium containing these two amino acids, harvested, and resuspended at a cell density of 6×10^6 cells/ml in complete medium containing cycloheximide at a concentration of 100 μg/ml. 30 min later ^{3}H-adenine (196 μCi/ml) or ^{14}C-adenine (4 μCi/ml) was added and exposure continued for 120 min. Cells were harvested, washed, starved, and aliquots of the ^{3}H-labeled population mutagenized with 25 μM Etd Br either in buffer (A) or buffer and glucose (B) for 240 min. The extent of mutagenesis was 92% ρ^- and 94% ρ^-, respectively. The cells were then mixed with ^{14}C-labeled controls, incubated under the two sets of conditions in the absence of mutagen. Extraction of mtDNA was as described previously (10) and its

(34–36). Probably the activities described by Piñon (31) and Paoletti et al. (32) do not have the requisite properties: the latter produces extensive degradation of mtDNA to acid soluble products and is activated not only by Etd Br but by all intercalating dyes tested, including quinacrine. It may, however, form part of the sequence or complex of enzymes responsible for the final degradation of mtDNA induced by mutagens observed by many investigators. An intimate relationship between intramitochondrial ATP and predisposition to mutagenesis has also been suggested by Šubik et al. (37). An additional factor in the activation of these nucleases by the complex between mutagen and mtDNA might then be provided by a lipophilic center in the mutagen capable of penetrating into and interacting with some adjacent component – perhaps the inner membrane of the mitochondrion (7, 38, 39). This model suggests certain experiments that we are currently pursuing – until their results are available nothing much can be gained by further speculation.

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analysis was by sedimentation in linear, neutral gradients of sucrose (16–30%, containing 5 mM Tris, 5 mM NaCl, and 10 mM EDTA, pH 8.1) at 5°C for 18,000 rpm × 19 hrs in the SW 27 rotor of the Beckman Model L centrifuge. Sedimentation was from left to right in the diagram, which was obtained by forcing 50% sucrose through the pierced bottom of the tube and collecting 40 one ml fractions by means of an ISCO collector. Samples were then analyzed for DNA by plating and counting of radioactivity in a liquid scintillation spectrometer (10). Control (no mutagen), ^{14}C, continuous line; experimental (Etd Br), ^{3}H, dotted line. The arrow indicates the peak position of nuclear DNA in the same set of gradients, previously identified by equilibrium sedimentation in CsCl gradients. In the experiments shown, the recovery of total ^{3}H dpm was $\geqslant 87\%$, relative to controls which had been labeled with this isotope but not subject to mutagenesis (either with or without incubation in buffer for 4 hr) after extraction and sedimentation analysis. C. Absence of "Registration" with euflavine. The results of two parts of an experiment are shown. Labeling, extraction, and analysis were as described for Fig. 4A and B, but treatment (in buffer and glucose) was with 25 μM euflavine for 240 min without mutagenesis (solid line), or 25 μM Etd Br for 120 min, 75% ρ^- (broken line). Sedimentation was for 18.5 hr. The position of the nuclear DNA peak is again shown by an arrow for reference. The number of ^{14}C dpm placed on and recovered from the cells treated with euflavine was $\geqslant 95\%$ that of a control without mutagen. The pattern for this control as well as a ^{3}H control all coincided with the solid line. In a separate experiment (not shown) exposure to euflavine was in buffer alone, with identical results.

REFERENCES

1. Ephrussi, B., Hottinguer, H., and Chimènes, A. M., Ann. Inst. Pasteur 76:419 (1969).
2. Marcovich. H., Ann. Inst. Pasteur 81:452 (1951).
3. Slonimski, P. P., in "Adaptation in Microorganisms," Cambridge University Press, pp. 76–79 (1953).
4. Nagai, S., Yanagashima, N., and Nagai, H., Bact. Rev. 25:404 (1961).
5. Slonimski, P. P., Perrodin, G., and Croft, J. H., Biochem. Biophys. Res. Comm. 30:232 (1968).
6. Mahler, H. R., Mehrotra, B. D., and Perlman, P. S., Prog. in Molec. and Subcell. Biol. 2:274 (1971).
7. Mahler, H. R., and Perlman, P. S., J. Supramolecular Structure 1:105 (1972).
8. Goldring, E. S., Grossman, L. I., Krupnick, D., Cryer, D., and Marmur, J., J. Mol. Biol. 52:323 (1970).
9. Nagley, S., and Linnane, A. W., J. Mol. Biol. 66:181 (1972).
10. Perlman, P. S., and Mahler, H. R., Nature (London) New Biol. 231:12 (1971).
11. Perlman, P. S., and Mahler, H. R., Biochem. Biophys. Res. Comm. 44:261 (1971).
12. Hollenberg, P., and Borst, P., Biochem. Biophys. Res. Comm. 45:1120 (1970).
13. Whittaker, P. A., Hammond, R. C., and Luha, A. A., Nature (London) New Biol. 238:266 (1972).
14. Vidova, M., and Kóvaĉ, L., FEBS Letters 22:347 (1971).
15. Whittaker, F. A., and Wright, M., Biochem. Biophys. Res. Comm. 48:1455 (1972).
16. Slonimski, P. P., at Int. Symp. DNA of Eukaryotes, Port Cros, France, May 5–9, 1971.
17. Ephrussi, B., and Hottinguer, H., Nature 166:956 (1950); Nucleocytoplasmic Relations in Microorganisms, Clarendon Press, Oxford (1953).
18. Atwood, K., and Norman, A., Proc. Natl. Acad. Sci. U.S. 35:696 (1949).
19. Hudson, B., Upholt, W. B., Denning, J., and Vinograd, J., Proc. Natl. Acad. Sci. U.S. 62:817 (1969); Bauer, W., and Vinograd, J., Progr. in Molec. and Subcell, Biol. 2:181 (1971).
20. Löber, G., Z. Chem. 11:135 (1971).
21. Waring, M., J. Mol. Biol. 54:247 (1970).
22. Mahler, H. R., and Perlman, P. S., Molec. Gen. Genetics 121:285 (1973).
23. Waring, M., J. Mol. Biol. 13:269 (1965).
24. Bauer, W., and Vinograd, J., J. Mol. Biol. 33:141 (1968); 47:419 (1970).
25. LePecq, J.-B., and Paoletti, C., J. Mol. Biol. 27:87 (1967).
26. Paoletti, C., and LePecq, J.-B., J. Mol. Biol. 59:43 (1971); Biochim. 53:969 (1971).
27. Saucier, J. M., Festy, B., and LePecq, J.-B., Biochim. 53:973 (1971).
28. Löber, G., and Achtert, G., Biopolymers 8:595 (1969).
29. Müller, W., personal communication.
30. Müller, W., and Crothers, D. M., Studia Biophys. 24/25:279 (1970).
31. Piñon, R., Biochem. 9:2839 (1970)
32. Paoletti, C., Couder, H., and Guerineau, M., Biochim. Biophys. Res. Comm. 48:950 (1972).
33. Moustacchi, E., Mol. Gen. Genetics 114:50 (1971); Moustacchi, E., Mahler, H. R., and Perlman, P. S., in preparation.
34. Meselson M., Yuan R., and Heywood, J., Ann. Rev. Biochem. 41:447 (1972); Yuan, R., Heywood, J., and Meselson, H., Nature (London) New Biol. 240:42 (1972).
35. Clark, A. J., Ann. Rev. Microbiol. 25:437 (1971).
36. Tomizawa, J.-I., and Ogawa, H., Nature (London) New Biol. 236:75 (1972).
37. Subik, J. and Kolarov, J., and Kóvàc, L., Biochem. Biophys. Res. Comm. 49:192 (1972).
38. Gitler, C., Rubalcava, B., and Caswell, A., Biochem. Biophys. Acta 193:479 (1969).
39. Azzi, A., Fabbro, A., Santato, M., and Gherardini, P. L., Eur. J. Biochem. 21:404 (1971); Azzi, A., and Santato, M., Biochem. Biophys. Res. Comm. 44:211 (1971).
40. Mahler, H. R., and Perlman, P. S., Arch. Biochem. Biophys. 148:115 (1972).
41. Ogur, M., St. John, R., and Nagai, S., Science 125:928 (1957).