Molecular Mechanisms of Mitochondrial Genetic Activity

EFFECTS OF ETHIDIOUM BROMIDE ON THE DEOXYRIBONUCLEIC ACID AND ENERGETICS OF ISOLATED MITOCHONDRIA*

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SUMMARY

When isolated, respiring mitochondria of Saccharomyces cerevisiae are exposed to ethidium bromide (EtdBr) they are capable of catalyzing the following discrete reactions.

\[
\text{mtDNA} + \text{mEtdBr} \xrightarrow{\text{Me}^+} [\text{EtdBr}_m \cdot \text{DNA}'] \\
[\text{EtdBr}_m \cdot \text{DNA}'] + (\text{ATP}) \xrightarrow{\text{nuclease(s)}} \text{fragments} + \cdots \\
(\text{Reactions 1 } + 2) \text{ mtDNA} + m\text{EtdBr} + (\text{ATP}) \xrightarrow{\text{fragments} + \cdots} n\text{ATP} [\text{EtdBr}_m \cdot \text{DNA}'] \\
\]

In this scheme ([EtdBr_m \cdot \text{DNA}']) represents a novel, stable derivative of EtdBr, linked (probably covalently) to mitochondrial DNA fragments (DNA') which, as isolated, have a mean mass of 12.5 \times 10^6 daltons. This intermediate had previously been shown to be formed in vivo and in vitro with kinetics of appearance and degradation consistent with it being the first product in the mutagenic sequence known to be initiated by EtdBr.

Experiments using respiratory inhibitors and uncouplers suggest that Reaction 1 depends on an energized state of the inner membrane, while Reactions 2 and 3 are driven by ATP, either added externally or generated by oxidative phosphorylation. Simultaneously there is an enhancement of the hydrolysis of added ATP to ADP plus Pi (Reaction 4).

The phenanthridinium dye ethidium bromide was originally synthesized and studied as an effective trypanocide (1,2). More recently this compound has received an ever-increasing amount of attention as a specific probe and reagent for extrachromosomal genophores in bacteria (3), algae (4,5), fungi (6-9), protozoa (1,2,10), and mammalian cells (11-17).

We are interested in the highly specific and effective mutagenic action of EtdBr in facultatively anaerobic yeasts such as Saccharomyces cerevisiae. This mutagenesis eventually produces stable, respiration-deficient mutant strains exhibiting an extrachromosomal, non-Mendelian mode of inheritance, now known to have resulted from the deletion of variable, but large segments of their mtDNA (6-9,18,19). Although it is thus the molecule ultimately affected (and is known to be susceptible to conformational alterations by virtue of its ability to bind EtdBr in an intercalative mode (20-24)) mtDNA need not be the exclusive or even its primary target in the mutagenic sequence. The dye is also known to be bound strongly to mitochondrial membranes.

K, disrupts this link by providing an alternate pathway for the activation of ATPase and leads to an immediate cessation of Reaction 2. Ethidium, a known antagonist to EtdBr in vivo has no effect on any of the reactions by itself, but blocks Reactions 2, 3, and 4 when added simultaneously with or subsequent to EtdBr.

Strains of S. cerevisiae known to be resistant to mutagenesis by EtdBr all exhibit a lowered rate of Reaction 3 but for different reasons: some exhibit a lowered capacity to form [EtdBr \cdot \text{DNA}'], others to bring about its degradation. Obligately aerobic, petite negative yeasts are known not to be affected by EtdBr in a permanent fashion: mitochondria of all these strains show a complete inability to catalyze Reaction 1.
In this scheme [EtBr\textsubscript{m}.DNA\textsuperscript{a}], abbreviated from now on as EtBr\textsubscript{m}-DNA, represents a novel, stable derivative of EtBr, linked (probably covalently) to the mitochondrial DNA fragments with a mass distributed around a mean of 12.5 x 10\textsuperscript{8} daltons (37). More complete evidence for the involvement of ATP and the mitochondrial ATPase in Reactions 2 through 4 will be presented below. We also present data suggesting that EtBr\textsubscript{m} in the presence of mtDNA and ancillary enzymes acts as an effective uncoupler (Reaction 4). These and the other studies described have prompted us to postulate that the mitochondrial energy coupling device provides a tight and obligatory link between two of the principal functions of the mitochondrion; energy transduction by its membrane and genetic capabilities of its DNA.

**EXPERIMENTAL PROCEDURES**

**Yeast Strains Used and Conditions of Growth**—A summary of the strains of *S. cerevisiae* used, their characteristics and growth requirements are summarized in Table I. In addition we employed certain obligately aerobic yeast strains for a limited number of experiments. These were Hansenula wingei (strain 5) originally provided by Prof. T. D. Brock; Torulopsis utilis, from the culture collection of the Department of Microbiology, Indiana University and Kluyveromyces lactis provided by Dr. Harlyn O. Halvorson. Unless otherwise indicated all cells were grown at 30\textdegree C with vigorous aeration in RHT, a medium containing 0.2% yeast extract (Difco); 0.1% (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4}, 0.1% KH\textsubscript{2}PO\textsubscript{4}, 0.5% MgSO\textsubscript{4}.7H\textsubscript{2}O, and 0.5% NaCl with 3% Na lactate as a carbon source and harvested when they had reached a turbidity of 1.0.

**Isolation and Incubation of Mitochondria**—Cells were converted to spheroplasts, and mitochondria were isolated as described previously (34, 38) except for omission of the wash with EDTA. For

![Table I](https://example.com/table.png)

**Table I**

| Strain\textsuperscript{a} | Source | Genotype | Phenotype |
|-------------------------|--------|----------|-----------|
| IL-16-10B-2            | P. Slonimski | a, his\textsuperscript{c} | Wild type, standard strain used for most experiments |
| N123                   | E. Moustacchi | a, his 1 | Wild type for set below |
| uv\textsuperscript{p}  | E. Moustacchi | as above, \( p \) | UV-sensitive, EtBr resistant in \( p \) production |
| uv\textsuperscript{p}\textsuperscript{73} | E. Moustacchi | as above | UV and EtBr sensitive |
| D-243-4A               | R. Criddle | \( \alpha \), ade 1, lys 1 | Wild type for set below |
| 70/1                   | R. Criddle | as above, \( \rho \) | Resistant to oligomycin |
| 70/1/\textsuperscript{p}\textsuperscript{73} | U. Flury | as above | RD\textsuperscript{y} |
| 7140-5IC               | P. Perlman (Orig. from R. K. Mortimer) | arg 4-2 +/+/arg 4-17 diclonal chromosome VIII | wild type for strain below |
| 7C4                    | P. Perlman (Orig. from R. K. Mortimer) | as above, rec 8 | rec\textsuperscript{c}, EtBr resistant |
| 197/2                  | P. Perlman (Orig. from F. Sherman) | \( \alpha \), ade 2-1 | wild type for strain below |
| rad 6\textsuperscript{c} | P. Perlman (Orig. from F. Sherman) | as above, rad 6 (uv\textsuperscript{p} 6)\textsuperscript{c} | X-ray, UV-sensitive, EtBr resistant |
| 2180 A, B              | H. O. Halvorson (Orig. from S. Fogel) | as above | |
| 1101                   | H. O. Halvorson (Orig. from S. Fogel) | diploid from above | |

\textsuperscript{a} All haploid unless otherwise indicated.

\textsuperscript{b} \( \rho \), \( \rho \textsuperscript{0} \) = respiration deficient, but not \( \rho \textsuperscript{0} \).

\textsuperscript{c} For auxotrophic strains we added the required growth factor.

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(25, 26), and some of the effects reported as capable of modulating the rate and extent of mutagenesis (for reviews see Refs. 8, 9, 27) are most readily accommodated in terms of a model that ascribes a determinative function to the inner membrane (28). The same applies to the interpretation of the remarkable differences in requirements that distinguish the action of EtBr from that of other, structurally related, intercalating dyes such as the alkyl acridinium derivatives (29). At the molecular level the exposure of cells of *S. cerevisiae* to EtBr under mutagenic conditions is known to result in the inhibition of (a) replication of mtDNA (30, 31) and (b) its transcription (32, 33); (c) the scission of the DNA molecule in a symmetrical fashion to produce molecules with a mass (after isolation) of 12.5 x 10\textsuperscript{8} daltons (37). More complete evidence for the involvement of ATP and the mitochondrial ATPase in Reactions 2 through 4 will be presented below. We also present data suggesting that EtBr\textsubscript{m} in the presence of mtDNA and ancillary enzymes acts as an effective uncoupler (Reaction 4). These and the other studies described have prompted us to postulate that the mitochondrial energy coupling device provides a tight and obligatory link between two of the principal functions of the mitochondrion; energy transduction by its membrane and genetic capabilities of its DNA.
incubations (30°) we suspended mitochondria from 100 ml of cells in 5 ml of Buffer P (0.1 M sorbitol, 100 mM KH_{2}PO_{4}, 100 mM NH_{4}Cl, 10 mM MgCl_{2}, 10 mM Tris, and EtdBr (60 μM; unless otherwise indicated) where appropriate).

Assays—For the assay of mitochondrial ATPase (39) we used 0.10-ml aliquots of particles suspended in Buffer T which is similar in composition to Buffer P except for the omission of KH_{2}PO_{4} and an increase in sorbitol concentration to 0.2 M. Formation of the covalent modification product (EtdBr-DNA) was assayed as follows. After incubation of mitochondria with [3H]-EtdBr (5 μCi per ml) (34, 36), an aliquot (0.10 ml) was diluted to 0.30 ml with 0.10 M NaCl plus 0.05 M Na_{2} citrate and lysed by the addition of 0.1 ml of sodium lauryl sarcosinate (10 g/100 ml, Sarkosyl, Geigy). DNA was removed by hydrolysis with 0.2 ml of 2 N NaOH for 18 hours at 23°. The DNA was then precipitated by the addition of 0.7 ml of NaH_{2}PO_{4} plus 0.3 ml of trichloroacetic acid. After 4 hours at 4° samples were filtered through glass filters, washed once with 10% trichloroacetic acid, twice with absolute ethanol, placed into vials with scintillation fluid and counted (34). This procedure is specific for EtdBr bound to mtDNA (34, 37), and the amount of product measured by it agrees within ±5% with measurements on mtDNA isolated either on sucrose gradients or polylysine-Kieselguhr columns.

 Destruction of the covalent modification product was assayed by the disappearance of acid-precipitable DNA obtained either as described above or by the use of cells, the DNA of which had been labeled with [3H]adenine (31, 34, 37) (New England Nuclear, specific activity 55 mCi per mmole). Protein was determined according to Lowry et al. (40).

Materials—The sources of the special chemicals used were: EtdBr, ATP, PEP, pyruvate kinase and oligomycin were all obtained from Sigma Chemical Co., St. Louis, Mo.; CCCP was a gift from Dr. P. Heytler, E. I. duPont de Nemours Co., Wilmington, Del.; Dio-9 was a gift from D r. R. J. Guillory, University of Hawaii, Honolulu, Hawaii, and colicin K was kindly provided by Dr. S. Silver, Washington University, St. Louis, Mo. All other chemicals were reagent grade.

RESULTS

EtdBr Elicits Novel Series of Reactions in Isolated Mitochondria

Isolated mitochondria of a number of respiration sufficient strains of S. cerevisiae, whether haploid or diploid, can carry out both the formation and degradation of EtdBr-DNA (Reactions 1 and 2) (38). This is demonstrated with mitochondria of haploid strain IL-16, suspended in phosphate buffer, in Fig. 1A (thick lines). Since the latter reaction exhibits an absolute requirement for ATP, either applied exogenously or generated internally by oxidative phosphorylation (here with succinate), combination of Reactions 1 and 2 can be regarded as Reaction 3, a DNAse that depends on both, the presence of EtdBr and probably ATP. Such ATP-dependent enzyme activities using DNA as a substrate are well known in eukaryotes where they form part of systems responsible for the replication, repair, recognition and recombination of their DNAs (e.g. Refs. 41-44). These systems are frequently also capable, as a corollary, to bring about the converse reaction, and thus act as DNA-dependent ATPase, usually with the amount of phosphate liberated exceeding the number of phosphodiester bonds cleaved (e.g. Refs. 45 to 49). These considerations have prompted us to search for this aspect of the reaction as well. As can be seen from the data represented by thin lines in Fig. 1, A and B, the presence of EtdBr-DNA, or more simply (in susceptible strains) EtdBr added to mtDNA elicits a stimulation of the intrinsic ATPase and brings about a substantial hydrolysis of added ATPase (Reaction 4).

Concentration Dependence and Stoichiometry—In yeast, EtdBr exerts its spectacular biological effects in the micromolar concentration range (6-9, 27-29). This is also the range in which we have previously established a satisfactory correlation in vivo between the kinetics of mutagenesis and of formation of [3H]-EtdBr-DNA (34). In earlier experiments in vitro (37) we had felt constrained to use [3H]EtdBr at a relatively high concentration in order to make certain that the product formed would be of sufficiently high specific radioactivity. This, however, proved unnecessary. As shown in Table II even at 10 μM the dye is present in sufficient excess to permit Reactions 1 to 3 to proceed at maximal rates. With EtdBr in excess, the extent of Reaction 1, as well as the total amount of EtdBr taken up by mitochondria, is strictly proportional to the amount of mitochondrial protein (and hence of mtDNA) added. This is most clearly demonstrated by the experiment in the bottom half, which employed a set of isogenic strains (2180A and B (haploids), HOH (diploid)), previously used in this laboratory for precise determinations of the amount of mtDNA and mitochondrial mass per cell (50). As can be seen, the amount of EtdBr covalently bound to the mtDNA of the diploid is twice that bound to either of its isogenic

![FIG. 1. Kinetics and requirements for reconstituted mutagenic events in isolated mitochondria. A, mitochondria of IL-16 grown in the presence of 150 μCi of [3H]adenine were isolated and suspended in Buffer T. At t = 0, 100 μM [3H]EtdBr was added to the sample and DNA-associated, acid-precipitable radioactivity was determined as described (●, ○, ●). At Δt = 30 min (•, △, ·) EtdBr-DNA incorporation (thick lines) and ATPase activity (thin lines) were assayed. Total activity at t = 90 min correspond to 2.7 × 10^{4} dpm of [3H]adenine and 1.7 × 10^{9} dpm of [3H]. Base-line ATPase activity (ATPaseo) corresponds to 4.5 μmoles of P_{i} liberated × mg of protein^{-1} × 10 min^{-1}; protein concentration was 5.9 mg ml^{-1}. B, same conditions as above, except that addition was either reversed in order (i.e. sucinate at t = 0 and EtdBr at t = 60, ○, □, △) or simultaneous (i.e. both succinate and EtdBr at t = 0, ●, ▲, ▲).](http://www.jbc.org/)

![TABLE II Determination of m](http://www.jbc.org/)

| Strain | [3H]EtdBr incorporation into DNA | Nucleotides/ | mg of protein^{-1} | X | Protein concentration | mg/αlilug | dpm/αlilug |
|--------|-------------------------------|------------|----------------|---|----------------------|-----------|-----------|
| IL-16  | 200                           | 2.47       | 2840            | 118|
|        | 200                           | 1.23       | 1510            | 126|
|        | 200                           | 0.62       | 720             | 120|
|        | 200                           | 2.47       | 2850            | 120|
|        | 200                           | 2.47       | 2810            | 117|
|        | 10                            | 2.47       | 2790            | 116|
| X2180-IA | 50                           | 1.74       | 1970            | 117|
| X2180-IB | 50                           | 2.13       | 2420            | 117|
| HOH-2  | 60                            | 3.84       | 4380            | 116|
The latter parameter are corrected for the amount of Pi present at 60 min in the experimental and a control sample without EtdBr, and for any spontaneous hydrolysis of ATP in such a control in the course of 60 min; all of them equaled 1.4 ± 0.2 pmol. All values are for 20.8 mg of mitochondrial protein.

EtdBr.DNA (0.1 ml) and Pi liberated (0.5 ml). Values of the aliquots were removed at 90 and 120 min for the measurement of were used. EtdBreDNA formation was complete after 60 min; at that point the solution was made 1 mM in ATP and further aliquots were removed at 90 and 120 min for the measurement of EtdBr-DNA (0.1 ml) and Pi liberated (0.5 ml). Values of the latter parameter are corrected for the amount of Pi present at 60 min in the experimental and a control sample without EtdBr, and for any spontaneous hydrolysis of ATP in such a control in the course of 60 min; all of them equaled 1.4 ± 0.2 pmol. All values are for 20.8 mg of mitochondrial protein.

The data of Table II permit a calculation of m, the number of EtdBr molecules incorporated into mtDNA as a result of Reaction 1. Assuming a value for haploid cells of 1.2 μg of DNA × mg−1 of mitochondrial protein (8, 9, 50), a molecular weight of 5.0 × 10^6 for mtDNA (8, 9), and a specific activity of 5 mCi mmole−1 for the EtdBr used, the values calculated for m are of the order of 1 EtdBr molecule bound for each 120 deoxynucleotides. This corresponds to 5 nmoles ml−1 of suspension and suggests that even at 10 μm the dye is present in sufficient excess to saturate all available binding sites.

We have also determined the value of n, the number of molecules of externally added ATP that can become hydrolyzed in the course of Reaction 4. As shown in Table III, the ratio of n:m equals 800 and hence n = 6.7 per phosphodiester bond split. This type of “nonproductive” ATP hydrolysis appears to be the rule for the behavior of bacterial recombination and restriction nucleases (46, 49).

**Requirements for Reactions 1 through 3**—The data presented in Fig. 2 and Table IV clearly indicate that, with the standard, actively respiring, mitochondria used in this investigation, (i.e. obtained from early stationary cells, grown on a nonfermentable carbon and energy source, such as lactate) Reaction 1 does not require free ATP, either added to the medium or generated internally. Furthermore, this reaction probably does not even depend on the small amount of intramitochondrial ATP present at the start of the experiment since it is unaffected by the addition of excess glucose plus hexokinase and is resistant to atractylate, Dio 9 and oligomycin. However, since it is responsive to uncouplers, the reaction is not independent of the energy generating properties of the mitochondrial membrane.

In contrast, Reaction 2, and in consequence Reaction 3 as well, exhibits an absolute requirement for (a) a supply of internal ATP (or compounds in ready equilibrium with it; see below), capable of interacting with the membrane-bound system for energy transduction, and (b) EtdBr-DNA. This is shown by the results of experiments summarized in Fig. 3. The reactions in question can be initiated in two ways: (a) either with added

**Table III**

| Time (min) | EtdBr bound in DNA (dpm/mg protein) | Molecules EtdBr | Pi liberated (pmol) | Molecules ATP hydrolyzed per EtdBr molecule liberated (μmol) |
|-----------|-------------------------------------|-----------------|-------------------|----------------------------------------------------------|
| 60        | 2.33 × 10^4                        | 1.28 × 10^4     | 6.5 × 10^5        | 0.9                                                       |
| 90        | 1.15 × 10^4                        | 6.3 × 10^4      | 10 × 10^5         | 1.4                                                       |
| 120       | 4.90 × 10^5                        | 2.7 × 10^4      | 10 × 10^5         | 1.4                                                       |

**Fig. 2.** Kinetics of formation of the EtdBr containing modification product of mtDNA. IL-16 mitochondria, suspended in Buffer T, were incubated with 100 μM [3H]EtdBr in the presence or absence of inhibitors. DNA-associated, acid-precipitable EtdBr was assayed each 30 min as described. Control; □-□, + colicin K (10^-6 M); ●-●, + CCCP (10^-5 M); ×-×, + oligomycin (20 μg per ml) ■-■, + Dio 9 (20 μg per ml). Total counts incorporated in control sample correspond to ~1400 cpm.

**Table IV**

| Addition | EtdBr incorporated (cpm after 30 min) | Inhibition |
|----------|-------------------------------------|------------|
| None     | 715                                 | 0          |
| ATP-regenerating system | 575 | 91 |
| EDTA (50 mM) | 65 | 91 |
| KCN (3 mM) | 317 | 56 |
| DNP (10^-4 M) | 324 | 55 |
| CCCP (10^-4 M) | 410 | 43 |
| CCCP (10^-5 M) | 72 | 90 |
| Colicin K (10^-1 M) | 36 | 95 |
| Glucose (10 mM) + hexokinase | 711 | 1 |
| Glucose + hexokinase + oligomycin (20 μg/ml) | 662 | 8 |
| Glucose + hexokinase + oligomycin + CCCP (10^-4 M) | 248 | 60 |
| Oligomycin + CCCP | 308 | 57 |
| Atractylate (50 μg/ml) | 720 | 57 |

* EtdBr (100 μM) in buffer P; for other details see “Experimental Procedures.”
* All normalized to equal protein concentration.
* 1 mM ATP + 5 mM P-enolpyruvate + 5 enzyme units of PK.

For the purpose of this discussion we assume the specificities and modes of action commonly postulated in mitochondrial energetics (e.g. Refs. 51 to 55).
ATP or ADP, presumably by virtue of the presence of adenylate kinase in the intermembrane space of yeast mitochondria (56), or with GTP (or GDP), due perhaps to phosphate transfer by nucleoside diphosphate kinase; the resultant adenine nucleotides will then have to be transferred across the inner mitochondrial membrane by the transporter system (57-59); or (b) these reactions can be made dependent on the energy generating oxidation of members of the citric acid cycle. In the former case (initiation with ATP) the reactions are blocked by inhibitors of the adenine nucleotide transporter (atracylate); the F1-ATPase (Dio 9); its activation of the ATPase; oligomycin, Dio 9, and antimycin A (5 μg per ml); ▲ ▲ ▲, DCCP (10^{-5} M); ● ● ●, +CCCP (10^{-4} M); X X X, +oligomycin (20 μg per ml); ■ ■ ■, +Dio 9 (20 μg per ml); ▼ ▼ ▼, +malonate (100 mM); ○ ○ ○, +antimycin A (5 μg per ml); ▲ ▲ ▲, +atracylate (50 μg per ml).

**Fig. 3.** Kinetics of degradation of the EtdBr containing modification product of mtDNA. IL-16 mitochondria (3.1 mg per ml), suspended in Buffer T, were incubated with 100 μM [3H]EtdBr for 90 min. At this point, the sample was divided into various aliquots and inhibitors added as specified. After 20 min, 1 mM ATP (A) or 100 mM succinate (B) was added to the samples and DNA-associated, acid-precipitable EtdBr assayed 30 and 60 min after that. Total counts incorporated in control sample correspond to 1400 cpm. ○ ○ ○, control; △ △ △, +colicin K (10^{-5} M); ● ● ●, +Dio 9 (20 pg per ml); ▼ ▼ ▼, +malonate (100 mM); ○ ○ ○, +antimycin A (5 μg per ml); ▲ ▲ ▲, +atracylate (50 μg per ml).

**Fig. 4.** Effect of various agents on activation of ATPase by EtdBr-DNA. IL-16 mitochondria were incubated for 90 min in Buffer T with 100 μM [3H]EtdBr. At this point (t = -20), drugs were added to various aliquots of the sample and incubation continued for another 20 min, at which time 100 mM succinate was added to each sample (t = 0). ATPase activity was then assayed as described under “Experimental Procedures” using ATP as an added substrate. Base-line ATPase activity (ATPaseo) was 4.5 pmol/mg protein. The concentration of Pi liberated X mg of protein^{-1} × 10 min^{-1}; protein concentration was 3.1 mg per ml. O-O, control; △ △ △, colicin K (10^{-5} M); ● ● ●, +CCCP (10^{-4} M); X X X, +oligomycin (20 μg per ml); ■ ■ ■, +Dio 9 (20 μg per ml); ▼ ▼ ▼, +malonate (20 μg per ml).

**Identity of Requirements for Reactions 3 and 4**—Implicit in the presentation so far has been the assumption that Reaction 4, the relatively massive hydrolysis of added ATP represents in fact (or is at least activated by) another (i.e. the converse) aspect of Reaction 3. This assumption suggests that the ATP requirement of Reaction 2 is satisfied by and represents one part of the mitochondrial ATP synthetase or ATPase complex is the enzyme system implicated in both reactions. That this may be a valid hypothesis is indicated by the identity of the requirements and inhibition patterns, including their quantitative aspects, for the two reactions. As shown by a comparison of the data of Figs. 3B and 4, both of which deal with events driven by succinate-dependent oxidative phosphorylation, exposure of mitochondria to the uncouplers CCCP and colicin which prevents the formation of the covalent modification product (and of the DNAase) probably does so by virtue of their prior activation of the ATPase; oligomycin, Dio 9, and antimycin A block DNAse and prevent activation of the ATPase and finally, atracylate has no effect. It should also be emphasized that, although the concentrations of some of the inhibitors used, especially oligomycin and atracylate, appear high (even on a protein basis) compared to those commonly employed with mammalian mitochondria, they are comparable to or less than the ones reported in the literature to be effective with and utilized for particles from yeast (39, 50-59). There was no effect on any of the reactions of the solvents used to add the inhibitors.

However, frequently the strongest evidence concerning the validity of any biochemical hypothesis is provided by the use of appropriate mutants with a well defined phenotype. Below we present the results of studies on the various reactions with two classes of mutants: (a) those isolated for, and exhibiting, alterations in their EtdBr-DNAase as well as in their EtdBr-DNA-induced ATPase; (b) those with alterations affecting DNA repair and recombination. **Oligomycin-resistant Mutants Exhibit Same Pattern Also in Their EtdBr-DNAase as Well as in Their EtdBr-DNA-induced ATPase**—We have examined two representative mutants, both resulting in oligomycin resistance of their mitochondrial ATPase. While both also manifest a mitochondrial mode of inheritance of this trait, they differ in the nature of the primary lesion responsible. The first is a representative of a particular class of mitochondrial mutants isolated and characterized among others by Avner and Griffiths (63) and by Mitchell et al. (64). The strain used by us is mutant D-243-4A-OR-4, described by Shannon et al. (60). The other mutant (73/1) isolated in this
Fig. 5. Degradation of the EtdBr containing modification product of mtDNA in D243-4A and related strains (see Table I); effect of inhibitors.1 Mitochondria isolated from D243-4A (A), 73/1 (B), D243-4A-OR-4 (C), or 73/1/p2 (D) grown on 3% galactose were incubated in Buffer T with 100 μM [3H]EtdBr for 90 min. At this point, each sample was divided into aliquots and to each one inhibitors were added in the presence of succinate or ATP. Degradation (thick lines) and ATPase activity (thin lines) were followed as described. Total counts incorporated into each sample were A, ~1600 cpm; B, ~900 cpm; C, ~800 cpm; D, ~500 cpm. Base line ATPase activity (ATPase0) was expressed as micromoles of Pi liberated X mg of protein X 10 min X 1. Symbols correspond to the following additions at t = 0: O——O, succinate (100 mM); △——△, ATP (1 mM); V——V, ATP (1 mM) + antimycin A (5 μg per ml); ○——○, succinate + malonate (100 mM each); △——△, succinate (100 mM) + antimycin A (5 μg per ml); ×——×, ATP (1 mM) + oligomycin (20 μg per ml); ■——■, ATP (1 mM) + Dio 9 (20 μg per ml).

Recombination and Repair Mutants Show Altered Responses Not Only in Function and Degradation of EtdBr-DNA but Also in Their Induced ATPase—We have examined representatives of three classes of such mutants. Those in the first class, isolated by Moustacchi (67), exhibit a heightened susceptibility toward ultraviolet light in producing cytoplasmic ρ- mutants, without any effect on nuclear mutation rates. However, the two strains selected differ in both, the kinetics of their response to other mutagens such as bercnil (68) or EtdBr1 (27, 28), as well as in their mode of inheritance. The first, ρ0 evinces enhanced resistance to mutagenesis by these agents, as compared to 7123, its wild type parent, and exhibits a normal (nuclear) pattern of segregation. The second, ρ2 shows enhanced susceptibility to such mutagenesis and is abnormal, probably with a mitochondrial contribution to its inheritance. ρ2. Mutants in the second class are all nuclear in inheritance and rec- (recombination deficient) for nuclear markers. The particular examples (e.g. Z140-51C2C4) have been chosen for their enhanced resistance to mutagenesis by EtdBr1 (69). The last is an example of a class of nuclear mutants (rad), particularly susceptible to radiation induced damage both with x-ray and ultraviolet (70). The strain chosen was selected for enhanced resistance to EtdBr6. Results of some experiments are summarized in Fig. 6: Fig. 6A presents data relevant to the reactions with isolated mitochondria and Fig. 6B the rates of mutagenesis on intact cells under comparable conditions.

We observe in the first series that mitochondria of ρ0 appear normal in rate and extent of Reaction 1, the formation of EtdBr-DNA, the modification product. They are, however, completely incapable of degrading it and are also grossly deficient in eliciting the succinate or ATP-elicited stimulation of ATPase in response to its formation. We conclude that, (a) a nuclear mutation produces a defect in the ability of mitochondria to excise a particular form of damage produced in their DNA by EtdBr, and (b) that this deficiency is reflected simultaneously by an inability to produce the resultant stimulation of, an otherwise normal, mitochondrial ATPase.

The response of ρ2 mitochondria isolated from these two mutants, their parent wild type, as well as of 73/1-p-2, a ρ0 (mtDNA deficient) derivative obtained from 73/1 by means of prolonged treatment with ethidium bromide (6-9, 18, 66), are shown in Fig. 5, A to D. The wild type (Fig. 5A) exhibits the expected capabilities with regard to (a) Reaction 1, the formation of EtdBr-DNA; (b) Reaction 2, the degradation of this species coincident with (c) Reaction 4, the activation of ATPase; (d) Reactions 2 and 3 can be elicited either by succinate or ATP,
shown by \( \rho_0 \). Like the latter it exhibits a lowered rate of Reaction 3 but for a different reason. Relative to its wild type (Z140-51C) it forms an abnormally low amount \((<25\% )\) of the modification product \((see Fig. 6 legend)\). However, both its rate of formation and of its degradation, as well as the rate and extent of the resulting ATPase stimulation are normal. The results with \( \text{rad } 6 \) are even more spectacular. Under the conditions studied it is completely resistant to mutagenesis and in this regard resembles \( \text{rad } 5 \). However, in its lack of ability to incorporate EtBr into its DNA it resembles \( \text{rec } 6 \) but in an exaggerated fashion: the level found is \(<10\% \) that of the wild type \((53 \text{ versus } 580 \text{ cpm } \times \text{mg}^{-1} \text{ of protein})\).

These experiments lay the groundwork for an interpretation of the genetic results in molecular terms, and provide additional evidence of the tight coupling between the mtDNAse induced by EtBr and the resultant activation of the mitochondrial ATPase complex.

**Eufavine Prevents Reactions 3 and 4**—As mentioned in the introduction, eufavine is an intercalating dye similar in its structural aspects to EtBr and like it an effective mutagen \((Ref. 29 \text{ and citations therein})\). However, it must differ in its mode of action since its activity is restricted to the buds of dividing cells \((71)\). Furthermore, with starved cells in buffer we have shown it to act as a potent competitor in preventing the expression of the mutagenic action of EtBr \((27-29)\). With isolated mitochondria, eufavine, at concentrations used in these in vivo experiments \((25 \mu M)\), is completely ineffective in eliciting either the breakdown of (labeled) mtDNA or the activation of ATPase (Fig. 7). It is also completely incapable of preventing the formation of EtBr-DNA. However, once this compound is formed, the subsequent (or simultaneous) addition of eufavine leads to a complete protection against its ATP-induced breakdown, the ATP and EtBr-dependent DNAse, and the concomitant activation of ATPase. **Colicin K Acts as Uncoupler**—Certain bacteriocidins such as colicin K are believed to act by virtue of their ability to interfere with integrated membrane function of susceptible cells, perhaps by producing a configurational alteration that prevents proper energy coupling \((53)\). This mode of action resembles the one postulated for lipophilic uncouplers of oxidative phosphorylation such as CCCP. It therefore appeared of interest to compare the action of these two inhibitors in the current system. The data of Figs. 2 to 4 indicate that these two agents are in fact qualitatively similar, both in preventing the formation of EtBr-DNA and its degradation. This result is probably the consequence of an activation of the mitochondrial ATPase in a competitive fashion, presumably by altering the energy coupling device. Two corollaries emerge from these studies: \((a)\) that colicin K appears to be a highly effective uncoupler of mitochondrial energy transduction, an interesting effect that warrants further investigation, and \((b)\) that uncoupling by CCCP \((or \text{colicin})\) and by EtBr-

The fluorodervative FCCP is effective at concentrations ten times lower than those used with CCCP.
DNA requires the participation of the same mitochondrial entity and therefore appear to be antagonistic and perhaps even mutually exclusive.

Further corroboration of this hypothesis comes from two types of experiments also summarized in Fig. 8. In the first we show that when operational "uncoupling" by EtdBr-DNA has reached its final level, the addition of CCCP only produces a small additional effect; in the second, that the addition of CCCP, once degradation of EtdBr-DNA has already been initiated, but before it is completed, leads to an immediate cessation of this reaction, coincident with a substantial enhancement of the ATPase.

**Effects of Other Modulators**—In this section we examine the effects of a number of agents that have either been claimed to affect the extent of mutagenesis by EtdBr or that may reasonably be expected to do so (Table V). Among the first group are caffeine, 8-azaguanine (72) and galactose (73) both of which can decrease the rate of formation of EtdBr-DNA without any influence, however, on any of the other reactions. This effect of galactose has also been observed for strains D243-4A and 73-1, when we compared mitochondria from glucose and galactose grown cells.

**Fig. 8.** Effect of CCCP and colicin K on reconstructed mutagenic events in isolated mitochondria. IL-16 mitochondria were incubated with 100 μM [3H]EtdBr for 90 min. At this point, 100 mM succinate was added to the sample. After 10 min, 2 aliquots were removed and 10−4 M colicin K (□—□) or 10−4 M CCCP (V—V) were added. DNA-associated, trichloroacetic acid-precipitable total EtdBr (thick lines) and ATPase activity (thin lines) were assayed as described. At t = 30 min, another aliquot was removed, 10−5 M CCCP was added (Δ—Δ), and the same measurements were performed. Control sample (O—O) was also assayed for these two parameters. Total counts at t = 0 corresponded to 1500 cpm. Base-line ATPase activity (ATPase0) was 4.2 μmoles of Paseline X mg of protein−1 X 10 min−1.

**Table V**

| Sample | 1. EtdBr incorporated into DNA at t = 90 min | 2. EtdBr-DNA recovered 60 min after addition of succinate at t = 90 min | 4. Ratio of ATPase0 to ATPasea |
|--------|--------------------------------------------|---------------------------------------------------------------------|-------------------------------|
| Control |                                            |                                                                     |                               |
| + cAMP (10−4 M) | 710                                      | 26                                                                  | 3.9                           |
| + Cycloheximide (100 μg/ml) | 720                                      | 25                                                                  | 3.8                           |
| + Caffeine (2 mg/ml) | 718                                      | 25                                                                  | 4.0                           |
| + Galactose (100 mM)* | 717                                      | 24                                                                  | 3.9                           |
| + Galactose (100 mM)* | 358                                      | 26                                                                  | 3.9                           |

*The reduction in amount of EtdBr incorporated in galactose-grown cells of other strains examined also shows a 50% inhibition of Reaction 1.

has been demonstrated in this laboratory to affect mitochondrial mutagenesis by berenil (68). We have also examined cyclic 3':5'-AMP, which has been implicated in the modulation of catabolite repression in yeast (7, 76, 77) as it does in E. coli (80, 81). With isolated mitochondria, only the addition of galactose produces a significant effect, by reducing the extent, but not the rate, of formation of EtdBr-DNA without any influence, however, on any of the other reactions. This effect of galactose has also been observed for strains D243-4A and 73-1, when we compared mitochondria from glucose and galactose grown cells.

**Petite Negative Yeasts**—Yeast species are frequently classified as either petite negative or petite positive (7-9, 83, 84). The latter include *Saccharomyces* and related facultative anaerobes. Among the former are obligate aerobes such as *Candida*, *Torulopsis*, *Klugeromyces*, and *Hansenula*. In such strains EtdBr not only fails to induce any stable respiration deficient mutants, but, in spite of being capable of exerting its customary inhibition of mtDNA and RNA synthesis, the agent does not produce a permanent, irreversible loss of mtDNA even upon extended growth in its presence (83-86). One would therefore anticipate the mitochondria isolated from such strains to behave quite differently from those of *S. cerevisiae*. We have tested particles from *Hansenula vilgini*, *Torulopsis utilis*, and *Klugeromyces lactis*. The results obtained confirm earlier, less complete experiments with intact cells and suggest the complete absence of any of the Reactions 1 through 4. This failure is not due to some obvious alteration in the mitochondria obtained from these cells, either intrinsic or as a result of isolation, since the particles take up EtdBr and exhibit base-line mitochondrial ATPase levels as high or higher than those described earlier for *S. cerevisiae*. We are therefore probably justified in concluding that (a) occurrence of Reaction 1 is a prerequisite for all the other three reactions and EtdBr-DNA is an obligatory intermediate, and (b) the resistance of these cells to EtdBr is the direct consequence of this inability to form the intermediate and the resultant lack in the degradation of their mtDNA.

* B. D. Mehrotra, and H. R. Mahler, unpublished observations.

1. A. A. Luha, P. A. Whittaker, and R. C. Hammond, private communication.
 because they (a) lack the ability to both reduce and oxidize cytochrome c and (b) exhibit a lesion in the coupling device (determined operationally by the oligomycin resistance of their respiration deficient activity (in complete accord with the properties expected for the drive the reaction, which has retained its sensitivity to atractylade adenylate kinase (56), addition of ATP but not of ADP can phosphodiester bonds from mtDNA, or whether in addition or alternatively, it requires a more active participation of the whole mitochondrial enzyme complex (ADPase) for its function. And (c) that coincident with or as a consequence of b the mitochondrial ADPase becomes activated (and can be assayed with externally added ATP) in a manner reminiscent of the response of this system upon exposure to lipophilic uncouplers.

The coupling device (or if preferred, inner membrane integrity) is also implicated in the first step, and the most parsimonious, although by no means the only possible, assumption to reconcile b and c is that (with ATP as the driving force) they represent different aspects of the same molecular event, also linked to or through inner membrane components; b would then correspond to the actual reaction involving ATP in stoichiometric amounts, and c to its maximal capacity, measurable only in the presence of added ATP. The evidence for point c, that the enzyme complex responsible for reaction is the mitochondrial ADPase, rests on both, experiments with specific inhibitors and with mutants of sufficiently well defined phenotype. This evidence appears reasonable and utilizes a standard reaction involving the liberation of inorganic phosphate from added ATP by mitochondria previously or simultaneously subjected to the various treatments described. The evidence for its implication in point b is somewhat weaker. We know that externally added ATP can drive the reaction, but can oxidative phosphorylation using sucinate, phosphate, and the adenine nucleotide pool inside the mitochondria. The degradation of EtdBr-DNA is linked to the energy coupling device; the experiments with uncouplers and inhibitors, especially in the appropriate mutants leave little doubt of that. But what is not certain is whether this link is solely by virtue of a continuing and stoichiometric requirement for ATP for the excision of the EtdBr and the cleavage of phosphodiester bonds from mtDNA, or whether in addition or alternatively, it requires a more active participation of the whole membrane-integrated complex; this remains to be established. In preliminary experiments with mitoplasts, which have lost their adeylate kinase (56), addition of ADP but not of ATP can drive the reaction, which has retained its sensitivity to octacyclate (in complete accord with the properties expected for the transporter system (57)).

The behavior of the mitochondria of respiration deficient mutants such as 3/1 and the p− petites tested earlier (34) is puzzling within this context. They are respiration deficient because they (a) lack the ability to both reduce and oxidize cytochrome c and (b) exhibit a lesion in the coupling device (determined operationally by the oligomycin resistance of their ATPase). Yet they are capable of performing the whole sequence of reactions, albeit at reduced levels. These observations suggest that such mitochondria retain in their membranes all the components (at least in a rudimentary form) necessary for the four reactions and can perform them at the expense of ATP generated by glycolysis.

**Relations to Biological Phenomena**—The reactions described in this and our two related previous publications (34, 37) appear to provide a satisfactory molecular basis for the mutagenic action of EtdBr described in the introduction. All the events and steps observed in vivo have now been duplicated with isolated mitochondria. The results with the different mutation-prone and mutation-resistant strains of *S. cerevisiae*, as well as with the resistant, obligately aerobic yeast species, not only provide essential confirmation of the central hypothesis, but account in a consistent manner for their susceptibility to mutagenesis by EtdBr: resistant strains are characterized by a deficiency in the rate or extent of breakdown of mtDNA due to Reaction 3 (sometimes because of their inability to bring about Reaction 1) and hypermutable strains carry out the degradation at an accelerated rate. The hypothesis also provides a framework within which to find an explanation of one other puzzling set of observations: that lesions in the coupling device, either as a result of mutation, e.g. op-1 (pet 9) (87-89) or manipulation (90) affect the rate of mutation from p+ to p−. These results have frequently been discussed (87, 88, 90) in terms of an alteration of the inner membrane, and this may, in fact, constitute their ultimate cause. But it is equally reasonable to assume that a more immediate manifestation may be a change in the substrate requirement for Reaction 2 (and 3), resulting in a degradation of mtDNA no longer dependent on its prior interaction with EtdBr. A similar explanation may also be advanced for mutagenesis by cauliflower which is known to require cellular (29, 71), and hence mitochondrial, division and may at some stage of this process bring the mtDNA into a configuration similar to the one found in EtdBr-DNA. These questions, and whether an analogous compound can also be formed (in an AT) requiring reaction by berenil and allyl proflavine, mutagens known to require an energy source in vivo (29), are susceptible to further inquiry.

**Relation to Isolated Enzymes**—The most mystifying part of the scheme is Reaction 1 since it represents an unexpected and hitherto unrecognized reaction. What will have to be determined is whether it reflects the action of one or more enzymes or of a peculiar configuration of mtDNA perhaps conditioned by its attachment to an appropriate membrane site (91). Such sites are known to be determinative for the replication of circular bacterial chromosomes and plasmids (92, 93). The possibility also exists that ribonucleotide tracts in mtDNA (identified so far unambiguously only in animal cells (91, 94-96)) actually constitute the reaction sites; EtdBr has been reported to be capable of becoming attached to tRNA in vivo (97). The absence of the reaction in rad 6 and in petite-negative strains opens this problem to ready inquiry.

There is no lack of precedent in prokaryotes for ATP-dependent nucleases, capable of recognizing specific sequences or configurations in their DNA (e.g. Refs. 41-49). Many of these enzymes are of large size and complexity and (when functioning as ATPases) are capable of hydrolyzing ATP in great excess over the number of phosphodiester bonds cleaved (i.e. they carry out reactions analogous to Reactions 3 and 4). No such activity has been explicitly reported for mitochondria. The DNase activity studied by Faletti et al. (98) responds not only to EtdBr but also to euflavine, and it lacks the requirement for ATP. Clearly these properties exclude it from consideration as the sole enzyme responsible for Reaction 2 or 3, but not as a possible participant within a complex that may result in modification of both its specificity and its requirements. The results with the mitochondria of *us* p5 suggest that the protein affected by this lesion constitutes (part of) the DNase in question. If that is so this mitochondrial protein is of nuclear specification.
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Molecular Mechanisms of Mitochondrial Genetic Activity: EFFECTS OF ETHIDIUM BROMIDE ON THE DEOXYRIBONUCLEIC ACID AND ENERGETICS OF ISOLATED MITOCHONDRIA
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