DNA BIOSYNTHESIS IN MITOCHONDRIA:

DIFFERENTIAL INHIBITION OF MITOCHONDRIAL AND NUCLEAR DNA POLYMERASES BY THE MUTAGENIC DYES ETHIDIUM BROMIDE AND ACRIFLAVIN

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SUMMARY:
The DNA-intercalating dyes ethidium bromide and acriflavin strongly inhibit the enzymatic synthesis of DNA catalyzed by rat liver mitochondrial DNA polymerase. When compared with the nuclear polymerase, the mitochondrial enzyme is much more sensitive to these dyes. These observations are in accord with the well known effects of these dyes in producing cytoplasmic (mitochondrial) but not nuclear mutations in yeast and trypanosomes. A possible mechanism for the mutagenic effect of the dyes is discussed.

INTRODUCTION:

Ethidium bromide and some acriflavins, dyes which intercalate between the bases of DNA, are known to cause cytoplasmic mutations in certain organisms (1-8). In yeast, the mutation is expressed by the disappearance of mitochondrial respiratory enzymes (3,9,10). An examination of the mitochondrial DNA in a number of these petite mutants provides evidence for both a decrease in the amount of mitochondrial DNA (11) and an alteration in its base composition (12-14). In Trypanosomes, the kinetoplast shows an alteration in structure and a decrease in its DNA content (5-8). At low concentrations of these dyes, the mutagenic process is specific in that only cytoplasmic mutations occur; no nuclear mutations are observed. A possible mechanism for the mutagenesis could involve a direct effect on the organelle DNA polymerase reaction. The availability of purified rat liver mitochondrial DNA polymerase (15,16) provided an opportunity of testing this possibility.

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The results of these experiments show that acriflavin and, more effectively, ethidium bromide severely inhibit the activity of the enzyme. Moreover, when mitochondrial and nuclear DNA polymerases were compared, it was found that the dyes exerted a selective inhibitory effect on the mitochondrial enzyme; under optimal assay conditions for each enzyme, the mitochondrial DNA polymerase was 37-fold more sensitive than the nuclear polymerase to ethidium bromide and 11-fold more sensitive to acriflavin. These observations could offer at least a partial explanation for the selective mutagenicity of these dyes toward organelle DNA.

We have recently presented evidence that the mitochondrial and nuclear DNA polymerases of rat liver are distinct enzymes (15). The present results offer further evidence in support of this view.

MATERIALS AND METHODS:

Rat liver mitochondrial and nuclear DNA polymerases were purified from the isolated organelles by procedures slightly modified (17) from those described previously (15). The mitochondrial enzyme at this stage of purification (fraction Mt-III) is at least 150-200-fold purified from the isolated organelle and 600-800-fold purified based on whole liver as the starting material. The nuclear DNA polymerase (fraction Nc-III) is at least 75-fold purified from the isolated organelle and 900-fold based on whole liver.

Enzyme assays and other methods were performed as described previously (15). DNA concentrations were determined by the diphenylamine reaction (18) or by ultraviolet absorption. Calf thymus DNA was obtained from Worthington Biochemical Corp., deoxyribonucleoside triphosphates from Calbiochem or Sigma Chemical Co., and H^3-thymidine triphosphate (specific activity 15.7 C/mMole) from New England Nuclear Corp. Neutral acriflavin, a mixture of proflavin (2,8-diaminoacridine sulfate) and euflavin (2,8-diamino-10-methylacridinium chloride), was obtained from Sigma Chemical Co. Ethidium bromide, manufactured by Boots Pure Drug Co., Nottingham, England, was a gift of Dr. J. Vinograd.

RESULTS:

The inhibition of the mitochondrial and nuclear DNA polymerases as a function of ethidium bromide concentration is shown in Fig. 1. The mitochondrial and nuclear polymerase results illustrated in curves A and C were obtained under optimal assay conditions, that is, using single and

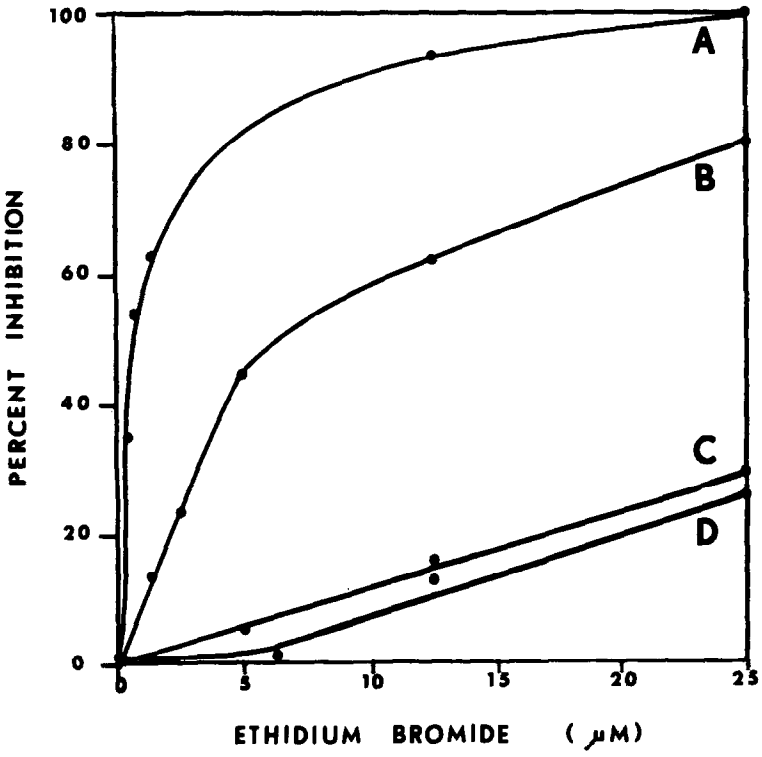

Fig. 1. Inhibition of Mitochondrial and Nuclear DNA Polymerase by Ethidium Bromide. The reaction mixtures consisted of 25mM Tris-HCl pH 8.0 (25°), 100 μg/ml calf thymus DNA, 0.015mM each of dATP, dCTP, dGTP and H^3-TTP (specific activity 450 mc/m mole), 7.5 mM Mg acetate, 5mM B-mercaptoethanol, 0.5mM EDTA, and either 0.15M NaCl (mitochondrial assay) or 0.025M KCl (nuclear assay). The reactions were run in a final volume of 125 μl which contained 25 μl of enzyme (15 μg Nc-III or 10 μg Mt-III). The concentration of ethidium bromide was as given in the figure. The tubes were incubated 1 hr. at 37° and acid-insoluble radioactivity determined by a slightly modified filter paper disk method (22). 100% activity was equivalent to 93 μμmoles for A, 36 μμmoles for B, 137 μμmoles for C, and 72 μμmoles for D. Curve A: mitochondrial enzyme, denatured DNA primer; curve B: mitochondrial enzyme, native primer; curve C: nuclear enzyme, native primer; curve D: nuclear enzyme, denatured primer.

double-stranded DNA respectively**. Clearly, the mitochondrial enzyme is much more sensitive to the presence of ethidium bromide than is the nuclear enzyme. This is particularly evident at low concentrations of the dye (about 1 μM) at which there is virtually no effect on the nuclear polymerase reaction while the mitochondrial enzyme shows an inhibition of 50-60%.

Since different forms of the primer are normally used for the nuclear and mitochondrial enzyme assays, we have extended the above experiments to include all combinations of enzyme and primer. A comparison of curves B and C or B and D shows that the selective inhibition of the mitochondrial polymerase is observed irrespective of which primer is used. It is noteworthy, however, that although ethidium bromide is an intercalating dye which has been shown to bind preferentially to double stranded DNA (19), it inhibits the mitochondrial polymerase more strongly when denatured DNA is used as the primer. This raises the question of whether, in addition to its intercalating properties, the dye may inhibit by acting directly on the enzyme. Further work is required to substantiate this possibility.

Acriflavin also inhibits the two DNA polymerases, but appreciably higher concentrations of this dye are required than with ethidium bromide (Table I.). Here again, a selective effect is observed. Under optimal assay conditions, the mitochondrial polymerase is 11 times more sensitive than the nuclear enzyme to acriflavin; in comparison, a 37-fold difference is seen with ethidium bromide. As was found with the latter dye, the differential inhibition is observed regardless of which form of primer is used.

DISCUSSION:

The results show that the mitochondrial DNA polymerase reaction is con-

**Recent experiments using more highly purified enzyme preparations than were described initially (15) combined with studies on a wide variety of our own carefully prepared as well as commercial DNA samples, indicate a primer preference in favor of single stranded DNA for the mitochondrial DNA polymerase (17). The extent of this preference varies greatly with the integrity of the primer and the extent of contamination of the polymerase preparation with nuclease activity. The preference of the nuclear polymerase is for double-stranded DNA (15).

TABLE I

COMPARISON OF THE INHIBITORY EFFECTS OF ETHIDIUM BROMIDE AND ACRIFLAVIN ON RAT LIVER MITOCHONDRIAL AND NUCLEAR DNA POLYMERASES

ENZYME	PRIMER	CONCENTRATION OF DYE (μM) PRODUCING 50% INHIBITION	
		ETHIDIUM BROMIDE	ACRIFLAVIN
MITOCHONDRIAL	DENATURED DNA	1	11
MITOCHONDRIAL	NATIVE DNA	7	27
NUCLEAR	DENATURED DNA	54	127
NUCLEAR	NATIVE DNA	37	123

The assay conditions were as given in the legend to Fig. 1. except that acriflavin was substituted for ethidium bromide in some experiments.

siderably more sensitive to the inhibiting dyes used than is the nuclear polymerase reaction. The observations lend additional support to the previous conclusion that the nuclear and mitochondrial DNA polymerases of rat liver are different enzymes (15). The results further suggest the interesting possibility of using these dyes to probe the relationship between nuclear and mitochondrial DNA replication in higher cells.

Although the enzymes studied here are derived from rat liver, it is nevertheless intriguing that the differential inhibitory effect of the dyes on the two polymerases is in accord with their well known effect in producing cytoplasmic (mitochondrial) but not nuclear mutations in yeast. Moreover, the relative effectiveness of ethidium bromide and acriflavin in producing cytoplasmic mutations (4,20) parallels their relative effectiveness in inhibiting the mitochondrial DNA polymerase reaction. Thus, it is tempting to speculate that a selective inhibition of the mitochondrial DNA polymerase, such as observed here, could play a casual role in the dye-induced

cytoplasmic mutations. While a partial inhibition of the mitochondrial polymerase could result in the replication of incomplete DNA molecules (particularly when the inhibition results from dye intercalation) and thereby produce deletion mutations, it is somewhat more difficult to use this hypothesis to explain the small changes in buoyant density (and therefore, probably in base composition) of the DNA which have been observed (11,13,14). It is much more difficult to explain the large buoyant density shifts obtained (12,13). The buoyant density changes could more easily be accounted for by the suggestion (21) that the binding of the dye to the polymerase molecule alters the specificity of the enzyme so that it acquires a preference for a particular base or bases and inserts these contrary to the specifications of the template strand. Such a hypothesis can now be tested.

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REFERENCES:

1. Ephrussi, B., H. Hottinguer and A.-M. Chimenes, Ann. Inst. Pasteur, 76, 351 (1949).
2. Ephrussi, B., H. Hottinguer and J. Tavlitzki, Ann. Inst. Pasteur, 76, 419 (1949).
3. Ephrussi, B., Nucleo-cytoplasmic Relations in Micro-organisms, Clarendon Press, Oxford (1953).
4. Slonimski, P.P., G. Perrodin, and J.H. Croft, Biochem. Biophys. Res. Commun., 30, 232 (1968).
5. Riou, G., C.R. Acad. Sci., Paris, 265, 2004 (1967).
6. Riou, G., C.R. Acad. Sci., Paris, 266, 250 (1968).
7. Steinert, M., and S. VanAssel, J. Cell Biol., 34, 489 (1967).
8. Simpson, L., J. Cell Biol., 37, 680 (1968).
9. Slonimski, P., Ann. Inst. Pasteur, 76, 510 (1949).
10. Slonimski, P., and B. Ephrussi, Ann. Inst. Pasteur, 77, 47 (1949).
11. Tewari, K.K., W. Vötsch, H.R. Mahler and B. Mackler, J. Mol. Biol., 20, 453 (1966).
12. Mounolou, J.C., H. Jakob and P.P. Slonimski, Biochem. Biophys. Res. Commun., 24, 218 (1966).
13. Mounolou, J.C., H. Jakob and P.P. Slonimski in Biochemical Aspects of the Biogenesis of Mitochondria, E.C. Slater, et al., eds., Adriatica Editrice, Bari, Italy, p. 473 (1968).

14. Wintersberger, E., and G. Viehhauser, Nature, 220, 699 (1968).
15. Meyer, R.R. and M.V. Simpson, Proc. Nat. Acad. Sci., U.S. 61, 130 (1968).
16. Kalf, G.F. and J.J. Ch'ih, J. Biol. Chem., 243, 4904 (1968).
17. Meyer, R.R. and M.V. Simpson, to be published.
18. Burton, K., Biochem. J., 62, 315 (1956).
19. LePecq, J.-B, and C. Paoletti, J. Mol. Biol., 27, 87 (1967).
20. Marcovich, H., Ann. Inst. Pasteur, 81, 452 (1951).
21. Slonimski, P.P. - see round table discussion in Biochemical Aspects of the Biogenesis of Mitochondria, p. 475 (1968).
22. Bollum, F.J., J. Biol. Chem. 234, 2733 (1959).