DNA SYNTHESIS IN MAMMALIAN NUCLEI: INHIBITION OF DNA SYNTHESIS IN THE RESTING RAT LIVER NUCLEUS BY ETHIDIUM BROMIDE, PROFLAVIN AND ACTINOMYCIN D*

M. Anwar Waqar and Leigh A. Burgoyne

School of Biological Sciences, Flinders University of South Australia, Bedford Park, South Australia, 5042.

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SUMMARY : The DNA polymerase of resting rat liver nuclei has been used to study the mode of inhibition of DNA synthesis by ethidium bromide, proflavin and actinomycin D. The DNA polymerase is reversibly inhibited by all three substances and all three substances appear to have the same general mode of action. The inhibitors seem to have only a small effect on the activity of the enzyme when there is an excess of suitable DNA priming sites but have a relatively larger effect when priming sites are limited. The possibility that inhibition of nucleases might be responsible for the lack of primer sites or the failure to "path-clear" at the primer sites has been investigated.

We wished to carry out a comparative study of the effect of ethidium bromide, proflavin and actinomycin D on a mammalian DNA synthesizing system for two main reasons. Firstly they all interfere with the process of DNA synthesis *in vitro* and it was hoped that study of their mode of action might throw some light on the mechanism(s) of DNA synthesis. Secondly these drugs have been used *in vitro* in attempts to distinguish between nuclear and non-nuclear DNA synthesis (1) and we wished to study briefly the factors that determine the effectiveness of these drugs *in vitro* as it would help in the evaluation of studies of this type.

The system used for this study was resting rat liver nuclei prepared in the absence of Ca^{2+} and Mg^{2+} as previously described (2) as this system is relatively simple and has already been studied.

Materials and Methods - Proflavin hemisulphate was obtained from British Drug Houses and ethidium bromide was a gift from Boots Pure Drug Co.,

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[1]EGTA : Ethylene glycol-bis-(2-aminoethyl ether)-N,N'-tetraacetic acid.

Nottingham, England. Actinomycin D was purchased from Calbiochem. α-^{32}P-dTTP (1 Ci/mmole at date of synthesis) was prepared as described by Symons (3). Calf thymus DNA was isolated (4) and activated by a brief exposure to pancreatic DNase I. Micrococcus lysodeikticus and ^{32}P-labeled Escherichia coli B DNA was prepared by lysis of the organism, detergent treatment (4), pancreatic RNase A digestion and phenol extraction. DNA was saturated with dye or antibiotic by dialysing it against buffer A (2) containing the dye or antibiotic in the concentration to be used in the assay.

Nuclei were isolated in buffer A system, in the presence of EDTA and EGTA as described previously (2). DNA synthesis was measured at 37° as described before (2), in the absence of Ca^{2+} or with added Ca^{2+} as indicated in figure legends. Nuclear protein and DNA were estimated using the procedure of Lowry et al (5) and Burton (6) respectively.

Results and Discussion - Ethidium Bromide Inhibition of DNA Synthesis :

Ethidium bromide inhibited DNA synthesis in nuclei as shown in Fig. 1A. The inhibition appeared to be progressive with time and this progressive inhibition did not appear to be due to increased rates of destruction of substrates or progressive denaturation of the DNA polymerase as the system could be reactivated simply by addition of activated calf thymus DNA. Moreover if the activated calf thymus DNA was allowed to saturate with ethidium bromide and then added to nuclei which had already been inhibited with ethidium bromide, it still reversed the inhibition (Fig. 1A). The nearest-neighbour analysis showed that the reactivation was not due to nucleotidyl terminal transferase activity (7,8). In contrast to this, non-activated calf thymus DNA or M. lysodeikticus DNA did not reverse the inhibition. Thus the major effect of added DNA is not explicable in terms of the well known ability of native DNA to bind ethidium bromide because the activated DNA, although present as a dye-complex in a solution of excess dye, reversed the inhibition whereas dye-free native DNA that would be expected to bind ethidium bromide avidly, did not reverse the inhibition.

Inhibition was a function of nominal dye concentration and a function of the amount of nuclei present indicating that the levels of the free-dye must be appreciably lowered by adsorption to the nucleprotein under the conditions in our assay (Fig. 1B).

Proflavin Inhibition of DNA Synthesis : Proflavin behaved in a very similar fashion to ethidium bromide. It caused a progressive inhibition of DNA synthesis that was reversible by activated calf thymus DNA, even when saturated

with the dye but not by dye-free non-activated calf thymus DNA (Fig. 1C).

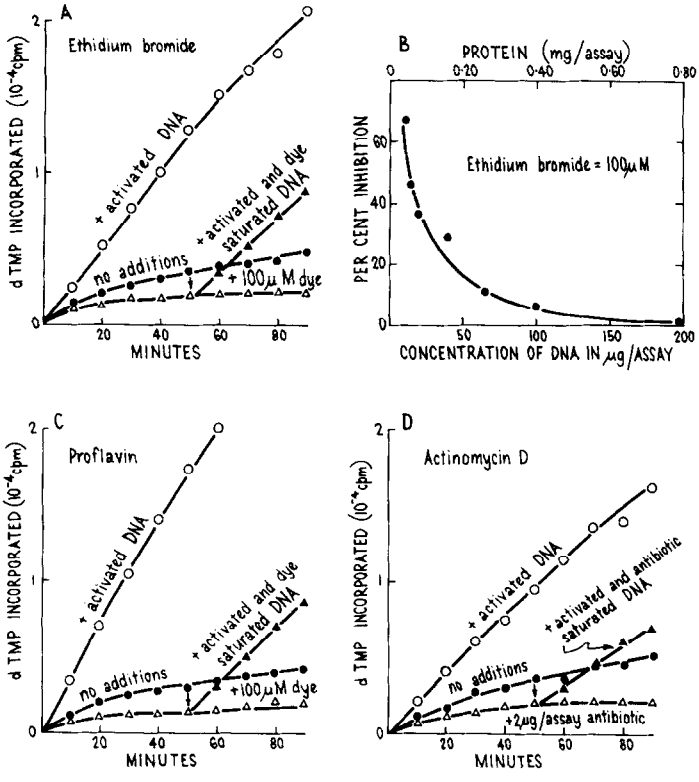

Figure 1. All assays were in 50 μl of Buffer A (2) - 0.34 M sucrose, 2 mM phosphoenolpyruvate, 1 mM EDTA, 0.2 mM EGTA, 10 mM $MgCl_2$, 0.4 mM ATP and 0.4 mM each of dATP, dCTP and dGTP. The nuclei were activated at 37° for 10 minutes as described previously (2).

A. Nuclei (1.57 x 10^6/assay) in assay solution with 20 μM α-^{32}P-dTTP (5.33 x 10^8 cpm/μmole): ●, no additions; Δ, + 100 μM ethidium bromide; ▲, + 100 μM dye added at 0 time and activated dye-saturated calf thymus DNA added after 50 minutes incubation; 0, + activated calf thymus DNA added at 0 time.

B. Inhibition of DNA synthesis measured as a function of nuclear concentration at a constant concentration of 100 μM ethidium bromide. Incubations were for 50 minutes and the α-^{32}P-dTTP had a specific activity of 9.37 x 10^8 cpm/μmole. 200 μg of DNA/assay was equivalent to 1.8 x 10^7 nuclei.

C. Nuclei (1.7 x 10^6/assay) in assay solution with 20 μM α-^{32}P-dTTP (4.62 x 10^8 cpm/μmole): ●, no additions; Δ, + 100 μM proflavin; ▲, + 100 μM dye added at 0 time and activated dye-saturated calf thymus DNA added after 50 minutes incubation; 0, + activated calf thymus DNA added at 0 time.

D. Nuclei (1.57 x 10^6/assay) in assay solution with 20 μM α-^{32}P-dTTP (6.88 x 10^8 cpm/μmole): ●, no additions; Δ, + 2 μg of actinomycin D; ▲, + 2 μg of actinomycin D added at 0 time and activated antibiotic-saturated calf thymus DNA added after 50 minutes incubation; 0, + activated calf thymus DNA added at 0 time.

Actinomycin D Inhibition of DNA Synthesis : Actinomycin D also behaved in a similar fashion to ethidium bromide in-as-much as it caused a progressive inhibition with time that could be reversed by actinomycin D-saturated-activated calf thymus DNA and this would seem to indicate a similar mode of action to ethidium bromide and proflavin (Fig. 1D). However there was one point of difference between actinomycin D and the other two inhibitors. Actinomycin D inhibition could be reversed by the addition of dye-free non-activated DNA from either calf thymus or M. lysodeikticus. This difference may simply be explained in terms of the relative affinity constants of ethidium bromide, proflavin and actinomycin D for DNA and nucleoprotein.

The progressive nature of these inhibitions and the response to drug saturated DNA, so long as it was pre-activated with pancreatic DNase I strongly suggested that all three drugs were interferring with the effectiveness of nuclear DNA priming sites and possibly with their production. The fact that the inhibition was progressive would also suggest that some chain elongation was necessary before the growth of the chain was suppressed by the dye.

Production of nuclear DNA priming sites is dependent on a Ca^{2+}-dependent endonuclease (2,9 & 10) and the "effectiveness" of the DNA priming sites could be dependent on a path-clearing exonuclease. The effect of ethidium bromide on the activity of nuclear nucleases was therefore examined briefly and the results are shown in Table 1. This type of experiment did not allow an accurate estimation of the endonuclease and exonuclease separately. However, the levels of nucleotides produced should be a crude index of exonuclease activity and the total levels of oligonucleotides and mononucleotides a crude index of endonuclease activity. This crude approximation was possible because, fortuitously, oligonucleotide levels were approximately constant over the range 0-100 μm ethidium bromide (Table I). At 100 μm ethidium bromide concentration where there was a just measurable inhibition of DNA synthesis, the calculated rate of exonuclease activity measured with free E. coli B DNA was found to become comparable to uninhibited DNA synthesis rates (Table I). However, this might be merely a coincidence because free E. coli B DNA may behave very differently to nucleoprotein DNA.

Thus the role of the endonuclease must remain unresolved at this time. The observed release of drug inhibition by drug-saturated-activated DNA strongly indicated that the presence of contaminating exonuclease activity would have a large effect on the apparent response to these drugs. Thus it is apparent that using these drugs for comparative studies between DNA and

Table 1. Effect of ethidium bromide on nuclear nucleases activity.

Assay conditions were as for Fig. 1. Nuclei (8.25×10^6/assay) were incubated with ^{32}P-Escherichia coli B DNA (4.62×10^6 cpm/mg) in the presence or absence of Ca^{2+}, Mg^{2+} and ethidium bromide as described below for a total time of 60 minutes. The products were separated by chromatography on DEAE paper using the two solvent system 0.25 M ammonium bicarbonate and then 0.3 M ammonium formate (11). At 100 μm ethidium bromide the DNA polymerase activity was inhibited 10%.

No.	Experimental Conditions	^{32}P-Radioactivity (cpm)	
		Oligo-nucleotides	Mono-nucleotides
1.	Complete	547	0
2.	" - Mg^{2+}	409	0
3.	" + Ca^{2+} (1 mM)	4,267	4,643
4.	" + Ca^{2+} (1 mM) + Ethidium bromide (10 μM)	5,558	1,575
5.	" " " " (20 μM)	7,256	1,448
6.	" " " " (50 μM)	5,631	1,135
7.	" " " " (100 μM)	5,478	764
8.	" " " " (250 μM)	1,877	0
9.	" " " " (500 μM)	261	0

partially purified DNA polymerases from nuclear and cytoplasmic sources may give results very difficult to interpret.

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