INHIBITION OF DNA-DEPENDENT DNA-POLYMERASE BY ETHIDIUM BROMIDE AND 3,8-DIAMINO-6-ETHYL-5-METHYLPHENANTHRIDINIUM BROMIDE

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1. Introduction

Ethidium bromide (EB) has been reported to interfere with the DNA-dependent DNA-polymerase catalyzed synthesis of DNA in vitro [1]. Since ethidium forms well-defined complexes with DNA the inhibition of the polymerase action may very well be the result of interaction between ethidium and template rather than a direct effect on the enzyme. In order to test this possibility, we undertook to study the inhibitory effect of this compound on the synthesis of DNA. We have also compared the results obtained to the corresponding data gathered using a structural analog of ethidium namely the 3,8-diamino-6-ethyl-5-methylphenanthridinium bromide (DEMB).

2. Materials and methods

Calf thymus DNA and deoxyribonucleotide triphosphates were obtained from Worthington Biochemical Corp., and [^{3}H]TTP (specific activity 54 Ci/mole) from Schwarz/Mann. DNA-polymerase I (EC 2.7.7.7.; 12.5 units/mg protein) and pancreatic DNase I (EC 3.1.4.5, 2000 units/mg protein) were purchased from Sigma Chemical Co. Ethidium bromide was obtained from Calbiochem. A crystalline sample of DEMB was kindly donated by Dr T. I. Watkins.

Binding of the drugs to DNA was determined as described previously [2]. Sample temperature was maintained at 37°C by means of a 10 cm jacketed cell and a constant temperature circulator.

The polymerase assay was essentially that of Bollum [3]. The reaction mixture (0.3 ml) contained: 0.4 M potassium phosphate, pH 7.0; 8 mM $MgCl_2$; 0.33 mg/ml BSA; 1 mM 2-mercaptoethanol; 0.1 mM each of dATP, dGTP, dCTP; 0.1 mM of [^{3}H]TTP (15 000 cpm/nmole); 0.072 mg/ml activated calf thymus DNA; 8 μg/ml enzyme and the indicated amounts of inhibitor. The acid-insoluble radioactivity after 30 min of incubation at 37°C was measured in a toluene-based solution.

Activation of the DNA template was carried out as described by Harwood et al. [4]. The method consists of partial digestion of the template with pancreatic DNase I (3.1 μg enzyme/mg DNA) for 10 min at 37°C.

3. Results

The inhibiting effects of EB and DEMB on DNA polymerase are shown in fig. 1a. In both instances significant inhibition of the enzyme is noted which increases with increasing concentrations of the drug. Specifically EB at an added EB to DNA-phosphate ratio of 0.20 produced an 83% inhibition of the polymerase. The same ratio of added DEMB to phosphate produced a 72% inhibition of the enzyme.

Although these data do not provide any information as to whether the drug interferes with the priming activity of DNA or whether it affects the enzyme directly, a distinction between these two possibilities can be made by examining the activity of the enzyme as a function of template concentration

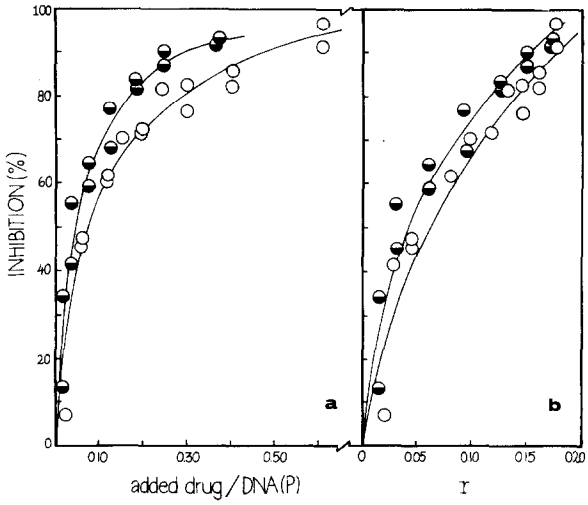


Fig. 1. – a) Inhibition of DNA-polymerase by EB (●) and DMEB (O) as a function of the added drug to DNA-phosphate ratio. – b) Inhibition of DNA polymerase by EB (●) and DMEB (O) as a function of the DNA-bound drug to DNA-phosphate ratio, r.

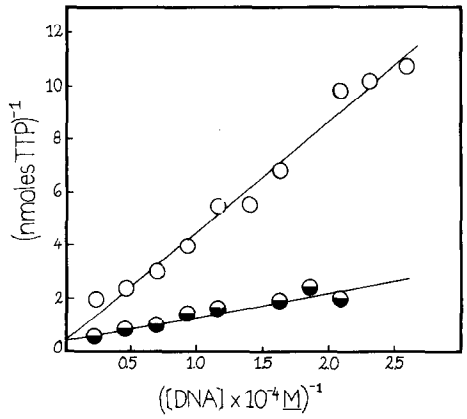


Fig. 2. Effect of the variation in the concentration of DNA template on the inhibition of TTP incorporation into newly synthesized DNA by EB. (●) control; (O), 4.3 X 10^{-6} M EB.

both in the presence and the absence of inhibitor.

The Lineweaver-Burk plot (fig. 2) suggests that the inhibition of the enzyme is related to the amount of template present in a competitive manner. Thus, it appears that the inhibitor has no effect on the enzyme itself but rather it interferes with the template activity of DNA. A similar conclusion has been reached previously concerning the inhibiting effect of EB on the DNA-dependent RNA polymerase-catalyzed synthesis of RNA [5].

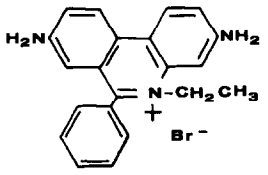
A comparison between the inhibitory effects of EB and DEMB on DNA polymerase should, therefore, be made on the basis of the amounts of DNA-bound inhibitor rather than the total amount of added drug. It turns out that, although the binding properties of EB and DEMB are comparable, EB interacts somewhat more strongly with DNA. Specifically EB is characterized by an intrinsic association constant K of 1.6 X 10^5 M^{-1} and DEMB by a K of 1.0 X 10^5 M^{-1} as determined from the corresponding Scatchard plots. Both values are obtained from measurements in 0.04 M potassium phosphate, 8 mM $MgCl_2$ buffer, pH 7; at 37°C, i.e. under conditions similar to those used in the assay for enzymic activity.

These data permit the results to be presented as percent inhibition vs. amount of bound inhibitor (fig. 1b). The obtained plot shows that the rate of DNA synthesis is decreased almost in proportion to the amount of bound inhibitor. It is also apparent that EB is a somewhat more effective inhibitor of DNA-polymerase than DEMB, although when the data are expressed in terms of bound rather than added drug the difference in the inhibiting effectiveness between EB and DEMB is decreased.

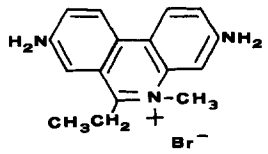
4. Discussion

Examination of the data shown in fig. 1b suggests that the DNA polymerase activity is almost totally abolished at a ratio of bound ethidium to DNA phosphate of about 0.18. The preferential stabilization of the double stranded conformation of DNA over the single stranded form of the polynucleotide, brought about by interaction with ethidium [6] is undoubtedly partly responsible for the inhibitory effect of the dye on the function of DNA-polymerase. A dissociation of the helix is required for replication and ethidium could interfere with this step.

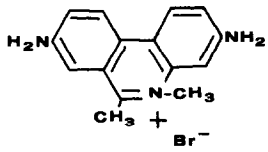
Alternatively the inhibition may result from the presence of bound ethidium molecules which restrict the length of DNA regions acting as templates to nucleotide sequences limited by neighboring drug



EB



DEMB



DDMB

Fig. 3. The structures of ethidium bromide (EB), 3,8-diamino-6-ethyl-5-methylphenanthridinium bromide (DEMB) and 3,8-diamino-5,6-dimethylphenanthridinium bromide (DDMB).

molecules. Ethidium is known to 'intercalate' i.e. become inserted between neighboring nucleotide bases in the double helix [7] and it appears that DEMB not only also intercalates but it may assume a conformation very similar to that of bound ethidium as well. This, at least is quite-likely in the case of 3,8-diamino-5,6-dimethylphen-anthridinium bromide (DDMB) which is structurally very similar to DEMB (fig. 3) and which forms complexes with DNA with circular dichroism properties identical to those of the DNA–EB complex [2]. Therefore, as the ratio of bound drug increases, the length of the regions available for replication may be decreased and a limit be reached at which these regions become too short to serve as effective templates.

The mechanism of inhibition, which is based on the notion that the intercalated drug molecule serves as a barrier in the attachment or the movement of the enzyme along the polynucleotide template, was previously suggested in order to explain the inhibitory effect of ethidium on RNA polymerase [5]. In view of the fact that ethidium is only a marginally more effective inhibitor of DNA-polymerase than the corresponding phenanthridinium derivative which lacks the C-6 phenyl substituent, the present results are consistent with but do not provide evidence for the above mechanism. However it is clear that since the conformation of the complex between DNA and these two derivatives is similar, the slightly stronger inhibitory effect of ethidium may be related to the presence of the bulky phenyl substituent, which projects into the major groove of DNA [7] and which may further impede the ability of the enzyme to become attached to or move along the template.

References

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