STUDIES ON THE EFFECT OF DISTAMYCIN A ON THE DNA DEPENDENT RNA POLYMERASE SYSTEM

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Summary: Addition of distamycin A to a DNA dependent RNA polymerase system blocks instantaneously the initiation of new RNA chains. Under the conditions used, elongation of growing chains is resistant to the action of the drug up to 4 minutes after addition of the antibiotic. Studies using density gradient centrifugation to follow the formation of the enzyme-DNA complex reveal that distamycin A inhibits the binding of the polymerase to DNA.

Introduction: Distamycin A (fig.1) is an antibiotic with marked antiviral activity against several DNA viruses (2-5). The antibiotic inhibits DNA dependent DNA and RNA synthesis (6-8). As shown previously, this inhibition is due to an interaction of the antibiotic with the DNA template (6-8). However, the detailed mechanism of the inhibitory effect of the drug remained unclear. Experiments were designed, therefore, to study more closely the effect of the antibiotic on the DNA dependent RNA synthesis. Evidence is presented now which indicates that distamycin A interferes with the binding of RNA polymerase to DNA.

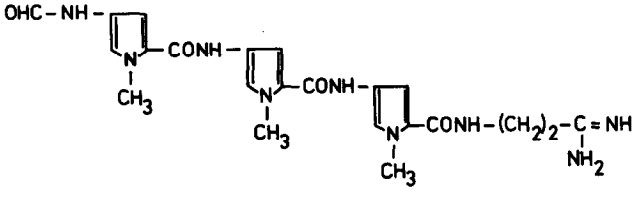

Fig. 1 Structure of distamycin A according to (1).

Material and Methods: Distamycin A was donated by Farmitalia, Milano, Italy. Adenosine-8-^{14}C-5'-triphosphate (59 mC/mmole), adenosine-5'-triphosphate-γ-^{32}P (650 mC/mmole) and thymidine-6-^{3}H (10 C/mmole) were purchased from the Radiochemical Centre, Amersham, Buckinghamshire, England. ATP, GTP, UTP, CTP and calf thymus DNA were obtained from Sigma Chem. Comp., St.Louis, USA. RNA polymerase was prepared according to Chamberlin and Berg (9). The incubation mixture described previously was employed for the assay (8). The reaction was stopped by the addition of 0.05 ml of 25 mM ATP in H_2O, 0.2 ml bovine serum albumin (3 mg/ml) and 8 ml 5% trichloroacetic acid. The precipitate was collected by centrifugation, solubilized in 0.4 ml of 0.5 N NaOH and precipitated with 8 ml 5% trichloroacetic acid. This step was repeated two more times. The washed precipitate was finally solubilized in 0.2 ml 0.5 N NaOH, 10 ml of the scintillator described previously (6) were added and the radioactivity measured in a scintillation spectrophotometer.

^{3}H-labelled DNA from SV40 viruses grown in the presence of thymidine-6-^{3}H was prepared according to Hirt (10). To examine the binding of the polymerase to SV40 DNA, 7.0 µg of enzyme and 0.2 µg of ^{3}H-labelled DNA were incubated for 5 min at 37°C in a binding buffer containing 10 mM Tris pH 7.9, 0.22 M NaCl, 5 mM mercaptoethanol, 5 mM $MgCl_2$, 0.1 mM EDTA, 0.12 ml total volume. 0.1 ml of the mixture was layered on top of a 5 - 20% sucrose gradient. For the preparation of the gradient, sucrose was solubilized in the binding buffer. After centrifugation at 246 000 x g for 150 min in a Beckman SW 56 rotor, 0.2 ml fractions were collected and after addition of 10 ml Instagel in H_2O (80% v/v) (Packard) the radioactivity of the fractions was determined in a scintillation spectrophotometer.

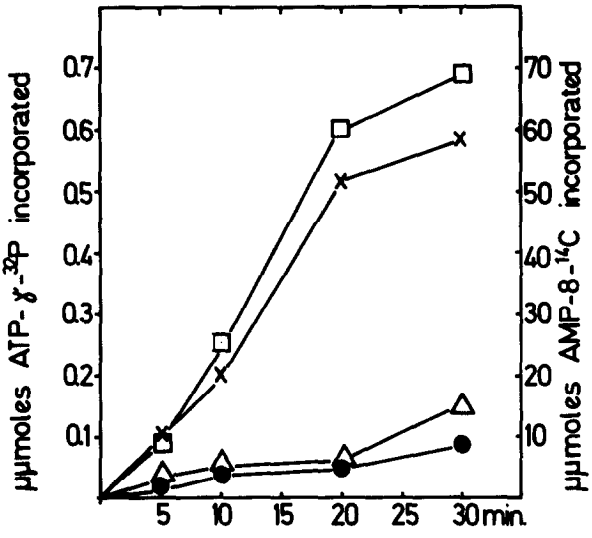

Fig. 2 Effect of 5×10^{-6} M distamycin A on the incorporation of (adenine-8-^{14}C)-AMP and γ^{32}P-ATP into RNA. □-□ ^{14}C-AMP incorporation (control); ●-● ^{14}C-AMP incorporation (plus distamycin A); x-x ^{32}P-ATP incorporation (control); △-△ ^{32}P-ATP incorporation (plus distamycin A). Distamycin A was added to the polymerase assay at zero time.

Results: The effect of distamycin A on the initiation of RNA synthesis was measured by incorporation of label from ATP-γ-^{32}P into the acid insoluble fraction of the RNA polymerase system. Total RNA synthesis was followed by the uptake of radioactivity from ^{14}C-ATP labeled in the adenine moiety. Fig.2 demonstrates that addition of the antibiotic at zero time leads to a strong inhibition in the uptake of both precursors. These results indicate that in the presence of distamycin A, fewer RNA chains are initiated. The observed decrease in ^{14}C-AMP uptake can be explained by the inhibition in chain initiation as the incorporation of both γ^{32}P- and adenine-^{14}C-labeled ATP is lowered to about the same extent. Considering the almost complete inhibition of the initiation at 5×10^{-6} M distamycin A it seemed probable that the inhibitory effect of the antibiotic on RNA synthesis occurs prior to the formation of the initiation complex.

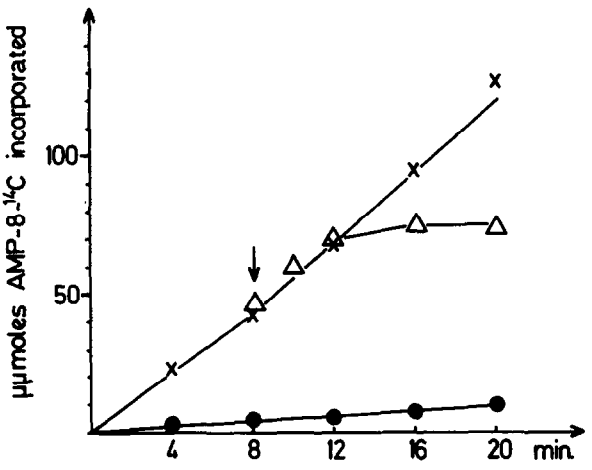

Fig. 3 Resistance of RNA synthesis to 10^{-5} M distamycin A after preincubation of the RNA polymerase system. x-x control; ●-● distamycin A added at zero time; △-△ distamycin A added 8 min after the start of the reaction.

If this is true, one should expect RNA synthesis to be resistant to the drug once initiation has occured. As fig.3 demonstrates this is indeed the case. Addition of distamycin A to the complete RNA polymerase system at zero time and subsequent incubation at 37°C for 4 min leads to a strong inhibition in the incorporation of label from ^{14}C-ATP compared to a control without the drug. If, however, the RNA polymerase system is preincubated at 37°C for 8 min, addition of the antibiotic is without any effect during the following 4 min period. With the data available so far, it cannot be decided if the inhibition observed after the 4 min interval is due to an interference with reinitiation or caused by a block in chain elongation. However, the data of figs.2 and 3 demonstrate that a major part of the inhibitory effect of distamycin A occurs before initiation of RNA synthesis.
A decrease in the number of RNA chains initiated can be due either to an interference with the binding of the polymerase to the template or due to an inhibition in the formation of the ini-

tiation complex. To examine the effect of distamycin A on the binding of the polymerase to DNA, advantage was taken of the marked difference in the sedimentation behavior of SV40 DNA and the enzyme SV40 complex[+]. Preincubation of RNA polymerase with SV40 DNA leads to the appearance of two new peaks corresponding to a material sedimenting faster than the free 13S enzyme (fig.4).

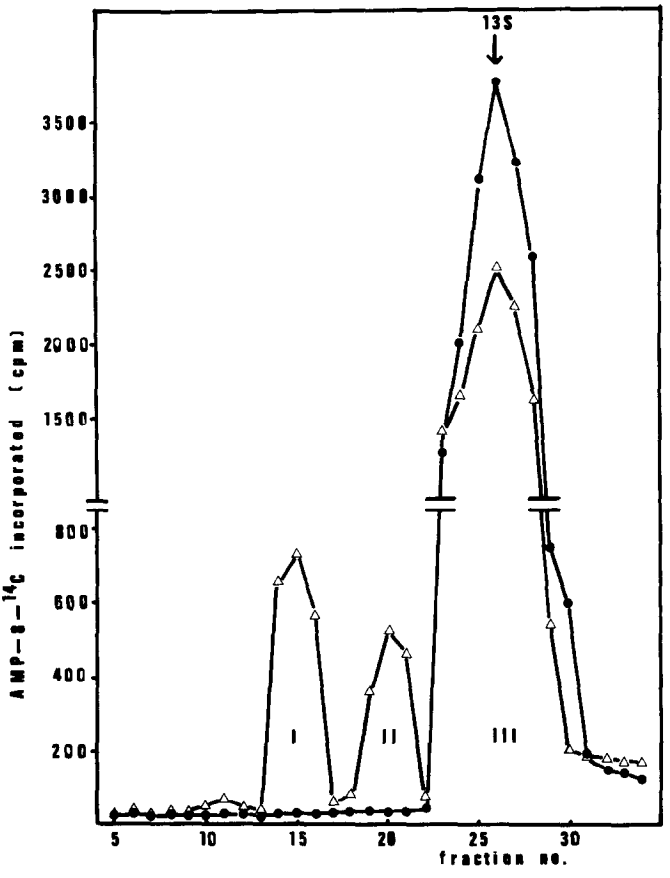

Fig. 4 Sedimentation behavior of E.coli RNA polymerase after incubation with SV40 DNA. ●-● polymerase; △-△ RNA polymerase preincubated with SV40 DNA. Incubation was performed with 0.4 µg SV40 DNA and 21 µg enzyme using the system described under methods. Enzyme activity was determined by following the uptake of ^{14}C-AMP after addition of 10 µg native calf thymus DNA to each fraction employing the assay described under methods.

[+] Studies on the effect of distamycin A on the RNA polymerase system with SV40 DNA (Component I and II) as templates yielded the same results as the assays pictured in figs.2 and 3 which contained calf thymus DNA.

The sedimentation behavior of ^{3}H-labeled SV40 DNA after preincubation with RNA polymerase is pictured in fig.5A. The bulk of the free DNA containing the supercoiled component I sediments as

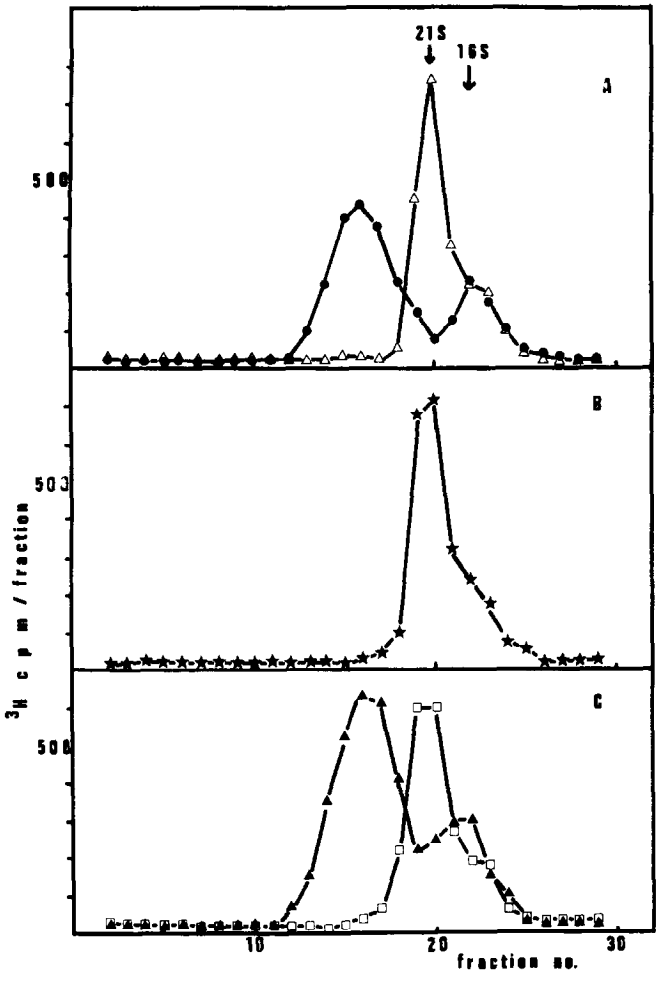

Fig. 5 Effect of distamycin A on the binding of RNA polymerase to ^{3}H-labeled SV40 DNA.
A: Sedimentation behavior of ^{3}H-SV40 DNA after incubation with E.coli RNA polymerase. △ -△ SV40 DNA; ●-● SV40 DNA preincubated with RNA polymerase.
B: Effect of distamycin A on the formation of the enzyme DNA complex. ★-★ DNA treated with 10^{-5} M distamycin A at 37°C, RNA polymerase was added after 5 min. Incubation at 37°C was stopped after a total period of 10 min. ATP and GTP (10^{-4} M each) were present throughout the incubation period.
C: Effect of distamycin A (10^{-5} M) on the sedimentation behavior of a mixture of DNA and RNA polymerase preincubated with or without ATP and GTP for 5 min at 37°C before addition of the drug. Total incubation time at 37°C was 10 min. ▲ -▲ distamycin was added after preincubation with ATP and GTP (10^{-4} M each); □ -□ distamycin was added after preincubation without ATP and GTP.

a narrow band at 21S (11). The small shoulder corresponds to the slower migrating 16S nicked circles (11). After incubation with RNA polymerase, the majority of the radioactive DNA exhibits the same sedimentation behavior as the RNA polymerase activity of peak I in fig.4 which shows the sedimentation of the enzyme after incubation with SV40 DNA. Fig.5B demonstrates that after treatment of the DNA with 10^{-5} M distamycin A no formation of the enzyme DNA complex can be observed. However, no effect of the drug can be seen if it is added after a preincubation of the DNA with the enzyme in the presence of the purine nucleoside triphosphates (fig.5C). Preincubation of DNA and enzyme alone does not protect against the action of the antibiotic (fig.5C).

Discussion: The studies presented here indicate that the inhibitory effect of distamycin A on RNA synthesis is mainly due to an interference with a step prior to the formation of the phosphodiester bonds. Studies on the complex formation between RNA polymerase and SV40 DNA revealed that the antibiotic affects the binding of the enzyme to the template DNA. Evidence has been presented recently for a binding of the E.coli RNA polymerase to AT rich sequences (12,13). Zimmer et al.(8,14) have shown that distamycin A preferentially binds to AT rich DNA. Furthermore Zimmer et al.(14) as well as Krey and Hahn (15) have demonstrated that a binding of distamycin A to DNA leads to a considerable conformational change of the DNA molecule. Both of these effects may interfere with the binding of the polymerase to the template whereas the elongation of initiated chains is less affected. In the RNA polymerase system preincubation without the drug has a protective effect against the action of the antibiotic. In accordance with these results there is no effect of the drug on the binding between DNA and the enzyme after a preincubation of the

DNA together with the polymerase and the purine nucleoside triphosphates. However, no enzyme DNA complex could be isolated after preincubation of DNA and enzyme alone and a subsequent addition of the drug. This may be explained by the fact that the binary complex between enzyme and DNA is less stable at the ionic strength used than the ternary initiation complex formed after addition of the purine nucleoside triphosphates (16,17). It is conceivable, therefore, that in contrast to the ternary complex, the binary complex dissociates rather rapidly to enzyme and free DNA which is than trapped by the antibiotic and now unable to reassociate. As an alternative one may assume that distamycin A is capable of splitting the less stable binary complex but unable to attack the stabilized initiation complex.

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