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The Mechanism of Inhibition of DNA Transcription *In Vitro* by Nitracrine (Ledakrin, C-283)

KRYSTYNA ŚLASKA, LESZEK SZMIGIERO, BARBARA JAROS-KAMIŃSKA, EWA CIESIELSKA AND MAREK GNIAZDOWSKI

Department of General Chemistry, Institute of Physiology and Biochemistry, Medical School, 90-131 Łódź, Poland

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SUMMARY

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In the presence of mercaptoethanol or dithiothreitol, an anticancer drug nitracrine (1-nitro-9,3-N,N-dimethylaminopropylaminoacridine, Ledakrin, C-283) forms irreversible complexes of decreased template activity with DNA. Up to 7 irreversibly bound [^{3}H]-nitracrine molecules were found per 10^4 nucleotides of calf thymus DNA in the presence of mercaptoethanol. Both average chain length and the number of polynucleotides decreased in the presence of the drug. It can be concluded that binding of the drug to DNA inhibits the initiation and elongation steps or induces a pre-early termination of RNA synthesis. On the other hand DNA bearing as much as 20 molecules of the drug per 10^3 of nucleotides (the complex formed in the presence of dithiothreitol) retains its ability to bind *E. coli* RNA polymerase.

INTRODUCTION

Nitracrine[1] (1-nitro-9,3-N,N-dimethylaminopropylaminoacridine) an acridine derivative with cytostatic activity and clinical application, inhibits macromolecular synthesis *in vivo* and in cell culture (see references 1 for early references and 2 for review). Covalent binding and cross-linking of the cellular DNA by the drug were reported by Konopa *et al.* (3) and recently confirmed by Filipski *et al.* (4). The drug forms intercalative complexes with DNA (4). Its inhibition of DNA-dependent RNA synthesis *in vitro* is low, however, unless sulfhydryl compounds are added to the reaction mixture (1, 5, 6). In the presence of either mercaptoethanol or dithiothreitol nitracrine forms complexes with DNA that are not dissociated during extraction with isobutyl alcohol and that exhibit low template activity (1, 6).

The aim of the experiments presented is to determine how this irreversible binding of the drug to DNA affects different steps of RNA synthesis. *E. coli* DNA-dependent RNA polymerase (EC 2.7.7.6) and calf thymus DNA were used in the study.

MATERIALS AND METHODS

[2,3,4-^{3}H]Nitracrine and the unlabeled drug were synthesized at the High Techni-

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[1] In previous publications the drug has been referred to under its original code "C-283" or its trade name Ledakrin (Polfa, Poland). The abbreviations used are: DNA_L and DNA_P, nitracrine-DNA complexes purified by extraction and dialysis, and similarly treated drug-free calf thymus DNA used as a reference template; SSC, 0.15 M sodium chloride in 0.015 M sodium citrate.

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cal School of Gdańsk, Poland. [γ-^{32}P]ATP and [γ-^{32}P]GTP were kindly provided by Professor P. Chambon, Strasbourg, France, or obtained from the Radiochemical Centre, England. Yeast tRNA and rRNA were gifts of Dr T. Wasiak and Dr W. Turski, Łódź, Poland. Other sources were: [^{14}C]ATP and [^{14}C]GTP (Radiochemical Centre, England); rifampicin, rifampin, and nonradioactive nucleoside triphosphates (Calbiochem, Switzerland); calf thymus DNA and DNA-ase I, code DPFF (Worthington, USA); 2-mercaptoethanol and Triton X-100 (BDH, England); dithiothreitol (Koch-Light, England); DEAE cellulose-DE-52, GF/82 and GF/C filters (Whatman, England); Sepharose 6B (Pharmacia, Sweden); bovine serum albumin, fraction V (Serva, West Germany); PPO and POPOP (Reanal, Hungary); and nitrocellulose filters, Synpor 5, 0.6 μm (Chemapol, Czechoslovakia). Other analytical grade chemicals were purchased from Cefarm, Poland.

E. coli RNA polymerase was isolated as previously described (1) but in some experiments DE-52 (instead of Cellex D) and Sepharose 6B (instead of Sephadex G-200) were used at the corresponding steps of purification without significant change of specific activity of the enzyme preparation.

Complexes of nitracrine with DNA were formed in the presence of mercaptoethanol and assayed in the *in vitro* transcription system after purification, or directly.

Purification of nitracrine-DNA complexes and determination of their stoichiometry and template activity. Purity of [^{3}H]nitracrine was assayed by thin layer chromatography. Eighty-four and eleven percent of the total radioactivity of the labeled preparation were recovered in spots of nitracrine and its water-insoluble hydrolysis product, 1-nitroacridone, respectively. [^{3}H]Nitracrine was diluted with the cold drug to specific activity of 3.2×10^9 cpm/mmole before use.

Complexes of nitracrine with DNA were prepared as previously described (1, 6) by incubation of calf thymus DNA (400–500 μg/ml) with either unlabeled or [^{3}H]nitracrine at the indicated concentration, in the presence of 15 mM mercaptoethanol or 5 mM dithiothreitol in Tris-HCl buffer, pH 7.5 for 1 hr. They were purified by extraction with isobutanol and dialysis. For the control assays DNA was similarly treated, but without drug addition (DNA_F). DNA was determined as described (6). 10 ml of toluene scintillator-Triton X-100 (2:1) mixture was added to 0.3 ml of [^{3}H]nitracrine-DNA solution and counted. As a reference, 0.3 ml of [^{3}H]nitracrine was mixed with an equivalent amount of DNA.

The template activity of DNA_L and DNA_F was assayed in the system essentially described by Burgess (7) but in which the KCl concentration was 0.05 M (1, 6). DNA concentrations are indicated in legends to figures and tables. [^{14}C]Labeled substrates of specific activities of 500–1000 cpm/nmole were used if not otherwise stated. To the reaction mixtures containing all the components in 0.15 ml *E. coli* RNA polymerase in 0.1 ml was added and the samples were incubated for 10 min at 37°. The reaction was stopped by addition of trichloroacetic acid-pyrophosphate, and the radioactivity measured, as described previously (1, 6) but a scintillation mixture containing 4 g PPO and 100 mg POPOP per litre of toluene was used instead of Omnifluor (in some experiments).

Estimation of the average chain length. The composition of enzyme assay mixture was the same as described (1, 6) except that albumin was omitted and DNA concentration was 40 μg/ml. Complexes of nitracrine with DNA were used without purification in RNA synthesis system. The reaction mixtures were prepared as follows (1) nitracrine (if present), DNA and mercaptoethanol (12 mM) were added to the other incubation components (Tris-HCl buffer, pH 8.0; $MgCl_2$, KCl, EDTA, nucleoside triphosphates, except [γ-^{32}P]ATP or [γ-^{32}P]GTP) and incubated in the dark at 20° for 1 hr in 0.14 ml. [γ-^{32}P]GTP (200–300 cpm/pmole) in 0.01 ml were added just before the enzyme. RNA synthesis was initiated by addition of 0.10 ml of RNA polymerase solution. After incubation (10 min, 37°) the enzyme was inactivated with EDTA, unlabeled ATP or GTP and trichloroacetic acid-orthophosphate-pyrophosphate were added and the precipitates were collected and washed on nitrocellulose filters (8).

Blanks without enzyme or without DNA were prepared. Their radioactivities were about 10–15% and 10–20% of complete controls. The latter of these blanks was subtracted.

Gel filtration of the transcript. DNA (80 μg) with nitracrine (0.1 mM) or without the inhibitor was incubated for 1 hr at 20° in 0.8 ml in the presence of mercaptoethanol (12 mM) and other components except the enzyme and substrates as described (1). Then ATP, CTP, UTP and [^{14}C]GTP (specific activity 20,000 cpm/nmole) in 0.1 ml and the enzyme (40 μg/assay) in 0.1 ml were added. After 10 min of incubation at 37° the reaction mixtures were cooled down to 0° and 125 μg of DNA-ase I (RNA-ase free) in 0.25 ml of 1 M NaCl was added. The samples were allowed to stand for 10 min. Then 300 μg of yeast tRNA in 0.15 ml of 0.1 × SSC, 0.03 ml of 25% sodium dodecyl sulphate, and 3 ml of phenol (saturated with water) were added. The tubes were shaken for 10 min at room temperature. To the water phase 2.5 volumes of cold ethanol (96%) were added, and the samples were stored overnight at −15°. The precipitates were washed three times with ethanol and dissolved in 0.3 ml of 0.1 × SSC. Three hundred micrograms of yeast rRNA in 0.15 ml and 0.05 ml of glycerol were added. The samples were applied to a Sepharose 6B column (0.9 × 45 cm) equilibrated with 0.1 × SSC. 1.5 ml fractions were collected (flow rate 0.15 ml/min) and absorbance at 260 nm was measured. Then 0.02 ml of 2.5% albumin and yeast rRNA (2 mg/ml stock solution) were added to each fraction to increase carrier RNA content to 60 μg per fraction followed by trichloroacetic acid-pyrophosphate. The precipitates were collected on GF/82 filters, washed and counted as above. Radioactivity of each fraction was expressed as a percentage of the radioactivity recovered up to emergence of the third peak (i.e., the acid-insoluble radioactivity of the fractions 8–24 was taken as 100%, see Fig. 3).

Binding experiments. Competition assays were done in a final volume of 0.25 ml at the concentration of reagents used as in the above described experiments on template activity assays or in a final volume of 0.1 ml.

In the first case DNA_F, DNA_L (formed in the presence of mercaptoethanol and 0.2 mM nitracrine) or both templates were mixed with the enzyme and all other components of the system except nucleotides. The missing template, where indicated, and substrates were then added, giving a final volume of 0.25 ml, and the samples were incubated for 10 min at 37°.

The assay mixture of final volume of 0.1 ml was identical to that of Mangel and Chamberlin (9). Buffer B was present in all solutions added during the experiment to ensure that the only change occurring in the samples was the addition of the component listed in the legends to tables and figures. Unless otherwise specified 1 μg of rifampicin was added per assay along with the substrates. The concentration of nucleoside triphosphates (0.4 mM), a higher specific activity of [^{14}C]GTP (about 10.000 cpm/nmole), absence of KCl in most experiments, and the shorter time of RNA synthesis (1.5 min) were the major differences between this system and that used in the standard conditions for template activity assay. The samples were precipitated with trichloroacetic acid, collected on GF/C filters, washed, and counted. DNA_L used in these experiments was formed in the presence of dithiothreitol and 0.2 mM nitracrine and purified by extraction and dialysis.

RESULTS

Stoichiometry of the irreversible binding of nitracrine to DNA. As already shown (5) exposure of DNA to nitracrine in the presence of sulfhydryl compounds affects its template activity for transcription. The inhibitory effect cannot be reversed by extraction of nitracrine-DNA complexes with isobutyl alcohol, followed by dialysis (1). Recent experiments indicate that the complexes are also resistant to treatment with octanol-chloroform mixture or phenol.[2] These results suggest that covalent binding may occur between nitracrine and the DNA template in the presence of thiols. The decrease of template activity plotted as a function of the molar ratio of irreversibly

[2] Unpublished observations.

bound [^{3}H]nitracrine to DNA, as well as the number of the inhibitor molecules bound at various nitracrine concentrations, are presented in Fig. 1. In the absence of mercaptoethanol the amount of labeled drug remaining associated with DNA after extraction and dialysis is only a few percent of that found in complexes formed in the presence of thiols. Assuming that no tritium atoms are lost during the attachment of the acridine ring to DNA, then very few nitracrine molecules are required to produce severe inhibition of template function. Two drug molecules bound per ten thousand nucleotides reduce the rate of RNA synthesis to about 20% (Fig. 1). At the highest nitracrine concentration used (0.5 mM) the number of molecules remaining in the complex was about seven per ten thousand nucleotides (see inset to Fig. 1).

Effect of nitracrine on the length of RNA product. Figure 2 presents the effect of nitracrine on incorporation of [γ-^{32}P]ATP and [γ-^{32}P]GTP, and on total RNA synthesis measured with [^{14}C]ATP and [^{14}C]GTP. The RNA chains retain triphosphate residues at their 5′ ends, and most if not all bear purine nucleoside triphosphate as the first 5′-residue. The decreased [γ-^{32}P]nucleoside triphosphate incorporation in the presence of nitracrine therefore indicates that the drug reduces the number of chains initiated. The number of ATP initiated chains has fallen by about 20% at a nitracrine concentration of 0.05 mM, while no further inhibition is observed at higher concentrations. The number of GTP initiated chains decreases stepwise within the range of drug concentrations examined. The results recalculated as indicated in the legend to Fig. 2 suggest that shorter RNA chains are synthesized in the presence of the drug (see the inset to Fig. 2). The average chain length, calculated to be about 2,000 nucleotides in the absence of the inhibitor, decreases by 50% at 0.2 mM nitracrine.

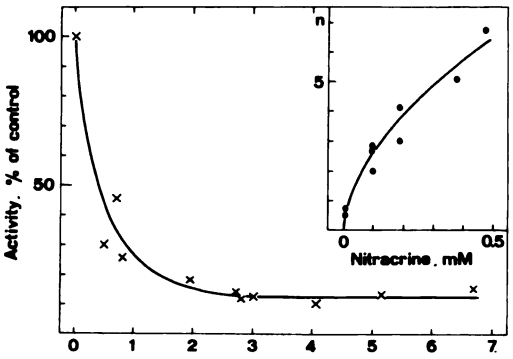

FIG. 1. *Inhibition of transcriptional template activity as a function of the number of [^{3}H]nitracrine molecules irreversibly bound per 10^4 DNA nucleotides (n)*

Nitracrine-DNA complexes were formed in the presence of mercaptoethanol, and purified. The template activity of DNA carrying different amounts of bound drug (n) was compared to that of DNA$_F$, as described in MATERIALS AND METHODS using [^{14}C]ATP as labeled substrate. The inset shows the number of drug molecules irreversibly bound at various nitracrine concentrations. The data of three experiments are given and each point corresponds to a separate DNA-nitracrine complex preparation. 10 μg of DNA and 12 μg of enzyme were added per assay. The incorporation of [^{14}C]AMP in the presence of DNA$_F$ was on average 2.0 nmoles.

The effect of nitracrine concentration on total RNA synthesis as measured by [^{14}C]AMP incorporation in these experiments is similar to the effect observed previously when nitracrine-DNA complexes were assayed without purification (1, 6). Higher inhibition is found when the complexes are formed and purified in the conditions used for determination of the stoichiometry (see Fig. 1 and ref. 1, 6). As pointed before (6) the efficiency of nitracrine binding in the presence of thiols could be influenced by DNA concentration and other components (Mg^{2+}, nucleoside triphosphates) present during formation of the complexes assayed directly without purification.

The effect of nitracrine on the chain length was also examined by gel filtration of the transcript (Fig. 3). RNA synthesized in the absence (Fig. 3A) or in the presence of nitracrine (Fig. 3B) was applied to a Sepharose 6B column along with carrier yeast rRNA and tRNA. The ultraviolet profile shows three consecutive peaks corresponding to rRNA, tRNA and substrates, which had presumably co-precipitated with macromolecular material during purification of the transcript (10). A larger amount of radioactivity was observed in the latter peak when the fractions were counted with-

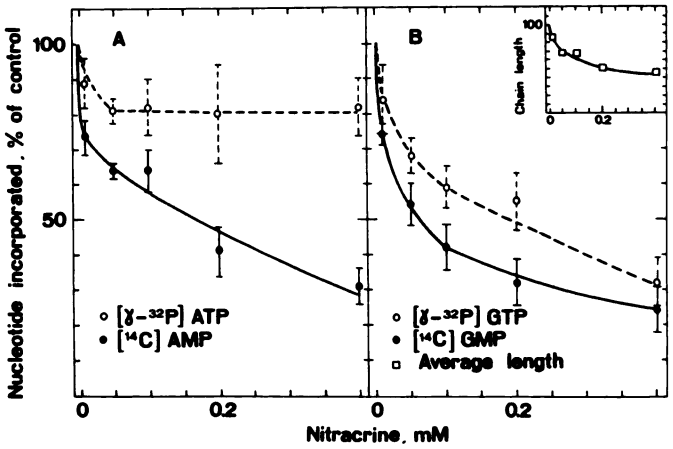

FIG. 2. *Effect of nitracrine on the average chain length of RNA*

Initiation [γ-^{32}P]ATP or [γ-^{32}P]GTP and overall RNA synthesis [^{14}C]ATP or [^{14}C]GTP were assayed in separate tubes in the same set of experiments. 30 μg of enzyme and 40 μg of DNA were used per assay. Average chain lengths were calculated using the formula:

$$\frac{([^{14}C]ATP + [^{14}C]GTP) \times 2}{[\gamma\text{-}^{32}P]ATP + [\gamma\text{-}^{32}P]GTP}$$

and expressed as percent of controls, i.e., assays without the inhibitor. Each bracket denotes the standard deviation of the mean value for four experiments. For three of them the specific activities of all labeled substrates were determined. The incorporation of [γ-^{32}P]ATP and [γ-^{32}P]GTP were on average 6.1 and 7.0 pmoles; [^{14}C]AMP and [^{14}C]GMP, 6.31 and 7.26 nmoles, respectively, and the average chain length was 2,050 in the control experiments. The inset shows the effect of nitracrine on average chain length expressed as a percent of control. For further details, see MATERIALS AND METHODS.

out prior trichloroacetic acid treatment (results not shown). In Fig. 3 the radioactive content of each fraction was measured only for acid-insoluble material. Hence only traces of radioactivity were detected in the last peak. In the three control experiments 77–80% of the acid-insoluble radioactivity was found in the high molecular weight region corresponding to carrier rRNA, while in the presence of nitracrine only 50–59% was recorded in this region, due to a shift to lower molecular weight.

The relative affinity of the enzyme for nitracrine-DNA complex versus untreated DNA. If the altered template has lost its ability to bind RNA polymerase, the enzyme should synthesize RNA at the same rate in systems containing either free DNA (DNA_F) or a mixture of DNA_F with purified nitracrine-DNA complex (DNA_L). If however DNA_L retains any ability to bind the enzyme, RNA synthesis in the mixed system should be inhibited. Our preliminary experiments indicated that the amount of RNA synthesized in a mixture of DNA_F and DNA_L was considerably lower (0.8 nmoles of [^{14}C]AMP incorporated) than that observed with the same amount of DNA_F alone (1.6 nmoles, see Table 1). This decrease in enzyme activity depends on the sequence of DNA addition, and on the temperature of incubation of RNA polymerase with DNA before initiation of RNA synthesis. The rate of RNA synthesis was similar when the enzyme was mixed with DNA_L, incubated for 3 min at 37°, and substrates then added with DNA_F (0.5 nmoles of [^{14}C]AMP) or without it (0.4 nmoles). When the enzyme was incubated with both or either template at 0° for 3 min, and the second template was added with the substrates, roughly the same amount of RNA was synthesized (0.7–0.8 nmoles, see Table 1).

To compare the relative affinity of the enzyme for DNA_L and DNA_F it seems proper to choose conditions such that: 1) transcription of the altered template is re-

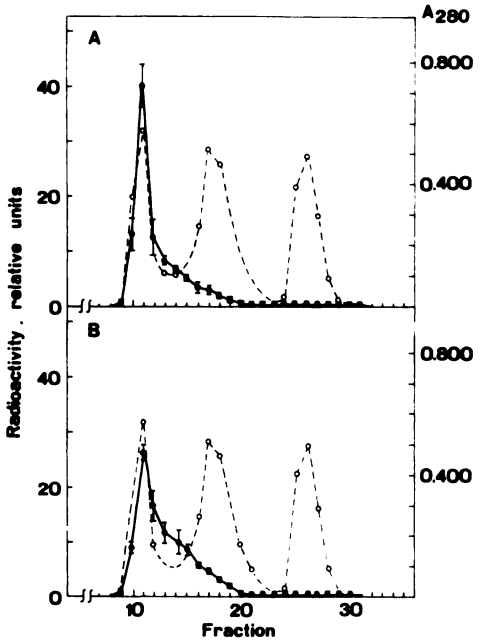

FIG. 3. *Effect of nitracrine on the elution pattern of transcripts from a Sepharose 6B column*

RNA was synthesized in the absence (A) or presence (B) of 0.1 mM nitracrine, purified and subjected to filtration in the presence of carrier yeast rRNA and tRNA. The acid-insoluble radioactivity in each fraction was expressed as a percentage of the radioactivity recovered in the fractions 8–24, i.e., up to the emergence of the third peak. The brackets denote the standard deviation of the mean acid-insoluble radioactivity in each fraction (full line) for three independent experiments. The amounts of RNA synthesized were on average equivalent to 13.0 nmoles of [^{14}C]-GMP in control experiments (A) and 5.2 nmoles in the presence of the drug (B). The ultraviolet profile (broken line) of one chromatogram is shown on each graph. The peaks of tRNA and the substrates appear respectively in fractions no. 17 or 18 and no. 26–28 of the eluate in other experiments. For further details, see MATERIALS AND METHODS.

duced to a minimum; 2) the activity of the enzyme is independent of DNA concentration; 3) the time of RNA synthesis is short enough to limit reinitiation; 4) exchange of enzyme molecules between templates during transcription is avoided. As the nitracrine-DNA complex formed in the presence of dithiothreitol exhibited the lowest template activity (1), it was used for this study. The number of drug molecules bound per one thousand nucleotides was 20 under these conditions of complex formation.

RNA synthesis was assayed in the conditions proposed by Mangel and Chamberlin (9) and used by Aktipis and Panayotatos (11) to investigate the effect of phenantridines on initiation of synthesis by bacterial RNA polymerase. The amount of RNA synthesized in the assays containing 2–8 μg of calf thymus DNA does not depend on DNA concentration (results not shown; see also Table 2, exp. I, where a twofold increase in DNA concentration has no effect on the transcription rate). The duration of synthesis (1.5 min) is not sufficient to complete one round of transcription on T_7 DNA (12). Although a heterogeneous population of polynucleotides is formed on calf thymus DNA (see Fig. 3), so that the time needed to complete one polynucleotide chain may vary, it can be assumed that limited reinitiation occurs under these conditions. To minimize the possibility of reinitiation by transfer of the enzyme from one template to another during RNA synthesis rifampicin (10 μg/ml) was added along with the substrates.

The kinetics of formation of the rifamp-

TABLE 1

Effect of DNA_L, formed in the presence of mercaptoethanol, on the transcription of DNA_F

The complex of nitracrine with DNA was formed at 0.2 mM nitracrine in the presence of mercaptoethanol, purified by extraction and dialysis. The composition of the preincubation mixture was as described for binding experiments (0.25 ml assay) except for the components listed in the table (ie. DNA_F or DNA_L) added with the substrates in 0.05 ml of Tris-HCl, 10 mM and EDTA, 0.05 mM and completing volume to 0.25 ml. 20 μg of DNA_F or/and DNA_L and 9 μg of enzyme per assay were used. For further details, see MATERIALS AND METHODS.

Components[a]	Components[b]	[^{14}C]AMP incorporated[c]	
		I	II
Enzyme, DNA_F	—	1.6	2.2
Enzyme, DNA_F	DNA_F	1.4	—
Enzyme, DNA_F, DNA_L	—	0.8	—
Enzyme, DNA_F	DNA_L	0.7	1.4
Enzyme, DNA_L	—	0.3	0.4
Enzyme, DNA_L	DNA_F	0.7	0.5

[a] Mix 3 min before assay and incubated at 0°(I) or 37°(II).

[b] Added with the substrates.

[c] Within 10 min; value given in nmoles.

TABLE 2

The effect of DNA_L formed in the presence of dithiothreitol on the transcription of DNA_F

DNA_F or DNA_L (each 4 μg) were incubated with the enzyme (2.1 μg) for 8 min at 37° in 0.05 ml, then nucleoside triphosphates and rifampicin (+RIF) in 0.05 ml were added and incubated for 1.5 min. Each experiment corresponds to separate preparations of DNA_L and DNA_F. DNA_L was formed at 0.2 mM nitracrine (0.16 mM in Exp. I) in the presence of dithiothreitol, and purified as described under MATERIALS AND METHODS. The fraction of enzyme transcribing DNA_F in the presence of DNA_L (f) was calculated using the formula:

$$f \times A + (1 - f) \times B = C$$

where A, B, and C are the activities of the enzyme in the presence of DNA_F alone, DNA_L alone, and the mixture of DNA_F and DNA_L, respectively. 2 × DNA_F–8 μg of DNA_F was used per assay.

Components mixed 8 min before assay and incubated at 37°	[^{14}C]GMP incorporated (1.5 min) in pmoles				
	I		II	III	IV
	−RIF	+RIF	+RIF	+RIF	+RIF
Enzyme, DNA_F	64	45	53	48	47
Enzyme, DNA_L	7	2	4	6	4
Enzyme, DNA_F, DNA_L	40	29	31	27	26
Enzyme, 2×DNA_F	—	45	—	—	—
Enzyme, DNA_L...DNA_F[a]	—	2	—	—	—
Enzyme, DNA_F[b]	—	421[b]	—	—	—
fraction of the enzyme transcribing DNA_F in the presence of DNA_L (f)	0.58	0.63	0.55	0.50	0.51

[a] Corresponds to the assays with DNA_L alone, but 4 μg of DNA_F was added 10 sec after the substrates.

[b] The assays containing 20 μg of DNA_F were incubated with 5.2 μg of enzyme for 10 min at 37° in a final volume of 0.25 ml.

icin-resistant enzyme-DNA complex is shown in Fig. 4. After 8 min of preincubation the activity of the enzyme when substrates are added along with 10 μg/ml of rifampicin is about 70% of that in the absence of the antibiotic. A plateau is reached after 30 min of preincubation. Closely similar results are obtained when the enzyme is preincubated with equal amounts of DNA_F and DNA_L (Fig. 4). This supports our assumption that the decreased RNA synthesis observed in the presence of DNA_L is due to the binding of some enzyme molecules to the inactive template, rather than to some indirect effect of DNA_L, e.g., an increase in viscosity.

In competition experiments, the fraction of enzyme transcribing DNA_F in the presence of DNA_L was calculated, relative to the rifampicin resistant enzyme activity with the DNA_F alone (Table 2). Qualitatively similar competition was detected in each case. Competition for RNA polymerase was assayed after mixing the enzyme with DNA_F or DNA_L and preincubating at 37° for the time indicated (Fig. 5). DNA_L or DNA_F respectively was then added and the samples further incubated for 8 min. Time zero corresponds to RNA synthesis assayed after 8 min of incubation of the enzyme with both templates. The fractions of enzyme transcribing DNA_F in both types of experiment were calculated. When

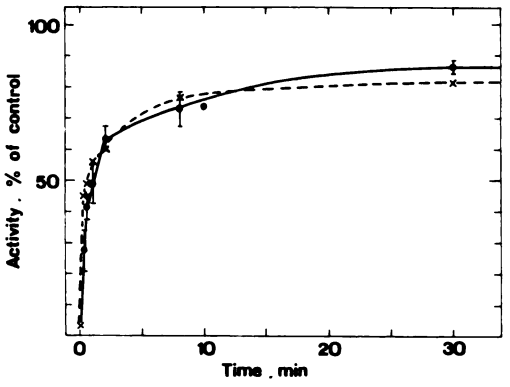

FIG. 4. *Kinetics of formation of rifampicin resistant complexes of RNA polymerase with calf thymus DNA (DNA_F) in the presence and absence of nitracrine-DNA complex (DNA_L)*

DNA_F (4 μg) in 0.04 ml, or a mixture of DNA_F (2.4 μg) and DNA_L (2.4 μg) in 0.06 ml, were preincubated with 1.7 μg of enzyme for the time indicated. Substrates in 0.06 or 0.04 ml were added along with rifampicin (1 μg) and the samples incubated for 1.5 min. The results are expressed as a percentage of control assays without rifampicin, incubated for 8 min before addition of substrates. The controls showed incorporation of about 50 pmoles (•DNA_F) and 35 pmoles (DNA_F + DNA_L) of [^{14}C]GMP. Data for DNA_F (●) are mean values from three independent experiments, and the brackets denote standard deviations from the mean. The curve (×) corresponding to rifampicin-resistant RNA synthesis with both templates (DNA_F + DNA_L) corresponds to one experiment. For further details, see MATERIALS AND METHODS.

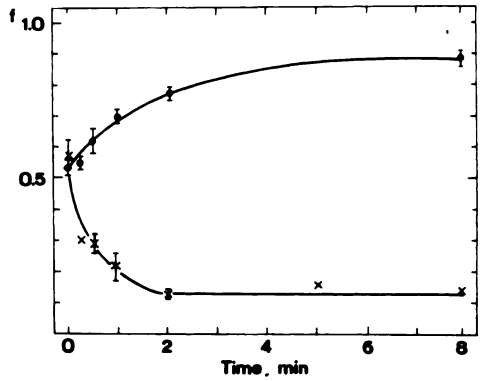

FIG. 5. *Rate of the complex formation between the enzyme and DNA_F or DNA_L*

DNA_F or DNA_L (each 4 μg) were incubated with 1.7 μg of enzyme for the time indicated (37°, 0.04 ml). Then 4 μg of DNA_L or DNA_F were added, and the samples incubated for a further 8 min before addition of substrates. Time zero corresponds to assays with enzyme added to the mixture of DNA_F and DNA_L and incubated in 0.06 ml for 8 min without substrates. Substrates ([^{14}C]GTP as labeled precursor) and rifampicin were then added and the samples (final volume 0.1 ml) incubated for 1.5 min. The fraction of enzyme transcribing DNA_F (f) was calculated as described in the legend to Table 2 and in the text. Figures in brackets denote the range of values from two independent experiments. ●——●, First template added to the enzyme: DNA_F; ×——×, first template added: DNA_L. f = 1.0 corresponds to RNA synthesis when enzyme was incubated with DNA_F for 8 min prior to addition of substrates with rifampicin and 4 μg of DNA_L.

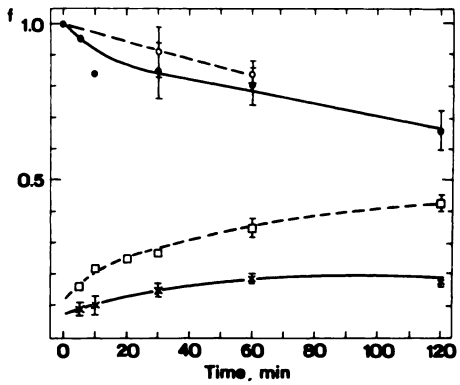

FIG. 6. *Stability of the complexes between enzyme and DNA*

Assay without KCl: The enzyme (2.1 μg) was incubated with 4 μg of DNA_F or DNA_L for 8 min in 0.04 ml, then DNA_L or DNA_F were added in 0.02 ml. RNA synthesis was then initiated. Zero time (f = 1.0) corresponds to the activity of enzyme incubated for 8 min prior to addition of DNA_L and DNA_F with the substrates. Figures in brackets show standard deviations for the mean results of three independent experiments. Enzyme incubated first with DNA_F, ●——●; DNA_L ×——×. *Assay in the presence of KCl*: The enzyme was incubated first with DNA_F (○- - -○) or DNA_L (□- - -□); then DNA_L or DNA_F in 0.03 ml with KCl to 0.1 M were added and the samples were further incubated for the time indicated, prior to addition of substrates. Other details are given under MATERIALS AND METHODS.

DNA_L was preincubated with the enzyme for 2 min, nearly 85% of RNA polymerase (f = 0.15) was unable to transcribe DNA_F subsequently added. On the other hand about 75% and 90% of the enzyme transcribed DNA_F when DNA_L was added to the polymerase-DNA_F mixture after 2 and 8 min respectively (Fig. 5). From the former series of experiments it may be concluded that after 2 min 85% of the enzyme attached to DNA_L. These results indicate that a shorter time is needed to form a complex of the enzyme with DNA_L than with DNA_F.

Once the enzyme is attached to a template it dissociates at a very low rate. The calculated fractions of polymerase transcribing DNA_F, after prior incubation of DNA_L with the enzyme for 8 min at 37°, increased from 0.08 to 0.2 during 1 hour of subsequent incubation with the two templates (Fig. 6). The remaining enzyme was tightly bound to DNA_L. The rate of dissociation increased when the preformed complex of the enzyme with DNA_L was transferred to higher ionic strength, and DNA_F added simultaneously. The fraction of enzyme transcribing DNA_F then increased from about 0.1 to over 0.4 after two hours.

The competition experiments indicated that a nitracrine-DNA complex exhibiting only a few percent of its initial unmodified template activity retained its ability to bind the enzyme.

DISCUSSION

Binding of nitracrine to DNA affects its template activity. Surprisingly few drug molecules seem necessary to inhibit RNA synthesis (Fig. 1). One possible explanation of this fact is that the drug binding sites are unevenly distributed and the drug binds preferentially to the initiation or transcribed regions of DNA. Alternatively, dur-

ing the reaction of nitracrine with DNA in the presence of thiols some tritium atom(s) are substituted, and this may lead us to underestimate the amount of drug bound.

Reaction of DNA with nitracrine alters various template functions to different extent. When a low number of drug molecules are bound both the number of initiated chains and their average chain length decrease. It has been recently shown that DNA-interacting drugs might decrease the average chain length of polynucleotides synthesized in the *in vitro* system, as assayed by the end-labeling technique, without affecting the elongation step of RNA synthesis. This effect has been shown when diacridines selectively inhibit transcription from some initiation sites (13). Gel filtration analysis of the product synthesized on the altered template confirms our suggestion that shorter chains are produced in the presence of the drug. The effect could be explained by an interference with the enzyme moving along the template during the synthesis of phosphodiester bonds, i.e., an effect on elongation rate. More probably it can be postulated that the drug molecules are so tightly bound to the template that the enzyme is unable to pass them and the premature termination of the polynucleotide chain results. The irreversibility of nitracrine-DNA complexes formed in the presence of thiols, and the high inhibitory effect found with a relatively low number of drug molecules bound to DNA, favors the latter explanation.

There is some preferential inhibition of GTP-initiated chains that might be ascribed to a specificity of drug binding. In fact it has been shown in our laboratory that poly A synthesis either on calf thymus DNA, or on poly dA:poly dT with ATP, as a sole substrate is insensitive to the drug (unpublished observation). On the other hand DNA bearing as many as 20 drug molecules per thousand nucleotides retains its ability to bind enzyme. This conclusion deserves comment. We are actually measuring the enzyme fraction forming a rifampicin-resistant complex with unaltered DNA, and indirectly estimating the fraction of enzyme binding to DNA_L. The similarity of the kinetics of complex formation with DNA_F and a mixture of DNA_F and DNA_L (Fig. 4), however, favors our assumption that there is "true" competition between the two templates for RNA polymerase. Both the rate of association of polymerase with the altered template and the dissociation of the DNA_L-enzyme complex when examined by competition do not change significantly. The dissociation rate of the complex with DNA_L increases however at higher ionic strength. This fact indicates that no irreversible inactivation of the enzyme occurs upon binding to nitracrine-DNA, and that the complex resembles the complex with free DNA.

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