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Inhibition of *Escherichia coli* RNA Polymerase by Bis(1-anilino-8-naphthalenesulfonate)†

Felicia Ying-Hsiueh Wu and Cheng-Wen Wu*,‡

ABSTRACT: Bis(1-anilino-8-naphthalenesulfonate) [bis(Ans)] is a dimer of 1-anilino-8-naphthalenesulfonate with a C-C linkage at the 4,4′ positions of the naphthalene rings. More than 90% of RNA polymerase activity is inhibited by 10^{-5} M bis(Ans), while no inhibition is observed with Ans at the same concentration. The concentration of bis(Ans) required for 50% inhibition is about 4×10^{-6} M using various DNA templates. The inhibition can be partially prevented by preincubation of the enzyme with DNA and/or nucleoside triphosphates. At 10^{-5} M, bis(Ans) has no effect on the binding of RNA polymerase to DNA as measured by retention of the enzyme-DNA complex on a nitrocellulose filter. However, little DNA retention was detected with 10^{-4} M bis(Ans). Kinetic studies and the differential effects of bis(Ans) on ^{3}H-labeled vs. γ-^{32}P-labeled nucleotide incorporations into the RNA product indicate that bis(Ans) at concentrations of $\leq 10^{-5}$ M selectively inhibits RNA chain initiation. Using T7 DNA as template, we have found that bis(Ans) inhibits the synthesis of the dinucleotide pppGpC catalyzed by RNA polymerase. Bis(Ans) binds to enzyme but not DNA as demonstrated by gel filtration and fluorescence spectroscopy. The binding of bis(Ans) to RNA polymerase has been studied by a fluorimetric titration technique. The binding isotherms show multiple binding sites of the dye, which can be divided into two distinct classes: a class of 16-18 strong binding sites with apparent $K_d = 2 \times 10^{-6}$ M and a class of 34-36 weak binding sites with apparent $K_d = 2 \times 10^{-5}$ M. Glycerol-gradient centrifugation analysis has revealed that RNA polymerase dimerizes in the presence of 1×10^{-4} M bis(Ans) but remains as a monomer at 1×10^{-5} M bis(Ans) in the high-salt (0.5 M KCl) buffer. Both the fluorescence and sedimentation results indicate that binding of bis(Ans) to the strong sites on enzyme inhibits RNA chain initiation, whereas binding to the weak sites induces dimerization of the enzyme concomitant with loss of its ability to bind DNA template. Further fluorescence studies show that aromatic amino acid residues are involved in the binding of dye.

The process of DNA-dependent RNA synthesis is rather complex and can be divided into several successive steps (Goldthwait et al., 1970): (a) association, the binding of RNA polymerase to the DNA template; (b) initiation, the binding of NTPs[1] to the enzyme-DNA complex followed by the formation of the first phosphodiester bond; (c) elongation, the subsequent addition of NTPs to form a polyribonucleotide chain; and (d) termination, the cessation of chain growth with the release of RNA product from the enzyme and DNA. Several antibiotics and dyes have been shown to inhibit specifically one or more of these steps by complexing with RNA polymerase. For instance, it has been proposed that rifampicin inhibits initiation (Hartmann et al., 1967; Wehrli et al., 1968; di Mauro et al., 1969), although it allows the continued synthesis of dinucleoside tetraphosphate by RNA polymerase (Johnston and McClure, 1976). On the other hand, streptolydigin affects both initiation and elongation (Siddhikal et al., 1969; Schleif, 1969). We have reported earlier that Rose Bengal preferentially inhibits RNA chain elongation (Wu and Wu, 1973), while Congo red (Krakow and von der Helm, 1970) and gallin (Liao et al., 1974) were found to block binding of RNA polymerase to DNA. All these inhibitors are important tools for elucidating the molecular mechanism of gene transcription.

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[1] Abbreviations used are: NTP, nucleoside triphosphate; Ans, 1-anilino-8-naphthalenesulfonate; bis(Ans), bis(1-anilino-8-naphthalenesulfonate); EDTA, ethylenediaminetetraacetic acid; WASP solvent, H_2O-saturated $(NH_4)_2SO_4$ (pH 8)-2-propanol in volume ratios of 18:80:2, respectively.

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Bis(1-anilino-8-naphthalenesulfonate) (bis(Ans)) (Figure 1) is a dimer of 1-anilino-8-naphthalenesulfonate (Ans) (Rosen and Weber, 1969). This dimeric form has a C–C linkage at the 4,4′ positions of the two naphthalene rings (Farris et al., 1977). Like Ans, bis(Ans) is virtually nonfluorescent in aqueous solution and becomes strongly fluorescent in nonaqueous solvents or when it is bound to hydrophobic sites in proteins (Weber and Laurence, 1954; Rosen and Weber, 1969).

Since bis(Ans) possesses the fluorescence characteristics of Ans and its chemical structure, first proposed by Rosen and Weber (1969), resembles that of Congo red, we have used bis(Ans) as a spectroscopic probe to study its interaction with RNA polymerase. The results presented here indicate that bis(Ans) inhibits RNA synthesis by interaction with RNA polymerase. Two classes of multiple dye binding sites on the enzyme have been demonstrated by fluorimetric titration studies. The strong binding sites are occupied at lower concentrations of the dye, which results in selective inhibition of RNA chain initiation. At higher concentrations of bis(Ans), the binding of the dye to the weak sites induces dimerization of the enzyme which loses its ability to associate with DNA template.

Experimental Section

Reagents. Unlabeled ribonucleoside triphosphates were purchased from P-L Biochemicals. ^{3}H- and ^{32}P-labeled ribonucleoside triphosphates were obtained from New England Nuclear Corp. Calf-thymus DNA was a product of Worthington Biochemical Corp. Unlabeled and ^{3}H-labeled T7 DNA were prepared from purified T7 phage essentially as described by Sadowski (1971). Bis(Ans) was synthesized by the method of Rosen and Weber (1969) and also obtained as a gift from Professor G. Weber. The concentration of bis(Ans) was determined by a molar extinction coefficient of 17×10^3 cm^2 mol^{-1} at 387 nm (Rosen and Weber, 1969).

RNA Polymerase. RNA polymerase was prepared from *Escherichia coli* K12 cells as described earlier (Wu and Wu, 1973). The enzyme is 95–98% pure as judged by the dodecyl sulfate-polyacrylamide gel electrophoresis and has a specific activity of 1000–1700 units per mg of enzyme (one unit is defined as nmol of the tritiated nucleoside monophosphate incorporated in 20 min). The concentration of protein was determined by the method of Bücher (1947), and using an extinction coefficient of $A_{280}^{0.1\%} = 0.65$ (Richardson, 1966).

Biochemical Assays. The enzyme activity was assayed by the procedure described previously (Wu and Wu, 1973). The standard reaction mixture (0.25 mL) contained 0.08 M Tris-HCl (pH 7.8), 10 mM $MgCl_2$, 4 mM β-mercaptoethanol, 0.4 mM each of ATP, CTP, GTP, and UTP (one labeled with ^{3}H, 5–8 $\times 10^3$ cpm/nmol), 0.03 μmol of calf-thymus DNA (or T7 DNA), and 2–5 μg of enzyme. The incubation was for 10 min at 37 °C. The reaction mixture was then chilled on ice followed by addition of 0.1 mL of 0.1 M sodium pyrophosphate and cold 5% trichloroacetic acid. The acid-insoluble material was collected on a glass-fiber filter (Whatman GF/C, 2.4 cm) and washed with cold 1% trichloroacetic acid and ethanol. The filter was dried and counted in a liquid scintillation counter.

The binding of RNA polymerase to DNA was assayed by retention of the complex on a nitrocellulose membrane (Freeman and Jones, 1967) using the modified procedure of Hinkle and Chamberlin (1970). In this procedure, the binding buffer which was used to dilute the assay mixture prior to filtration also contained the same concentration of bis(Ans) as that added to the assay mixture. The initiation of RNA chains was measured by the incorporation of [γ-^{32}P]ATP into acid-insoluble material using a slight modification of the method of Maitra and Hurwitz (1965).

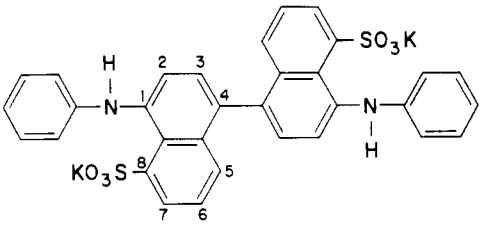

FIGURE 1: Structure of bis(Ans) [4,4′-bis(1-anilino-8-naphthalenesulfonate)].

The DNA-dependent synthesis of dinucleoside tetraphosphate catalyzed by RNA polymerase was measured as follows. The reaction mixture (0.1 mL) contained 0.05 M Tris-HCl (pH 7.8), 4 mM $MgCl_2$, 0.06 M KCl, 2 mM dithiothreitol, 8 μg of RNA polymerase, 40 μg of T7 DNA, 0.4 mM GTP, and 8 μM [^{3}H]CTP (2000 cpm/pmol) and was incubated for 25 min at 37 °C. The reaction was terminated by application of 10-μL aliquots to Whatman No. 3MM paper strips prespotted with 0.1 M EDTA. The papers were developed in the WASP solvent of Johnston and McClure (1976). After chromatography the chromatograms were dried, cut into 1-cm strips, and counted in Econofluor (New England Nuclear Corp.) in a liquid scintillation counter.

Sephadex G-75 Gel Filtration. To study the dye binding to RNA polymerase or DNA, the reaction mixture (0.2 mL), containing 3 mg/mL of enzyme (or 4 mg/mL of calf-thymus DNA) and 10^{-5} M bis(Ans) in 0.5 M Tris-HCl (pH 8), 0.2 M KCl, 0.5 mM dithiothreitol, and 0.1 mM EDTA, was incubated at 37 °C for 30 min. The mixture was then loaded onto a Sephadex G-75 column (1 × 14 cm) equilibrated and then eluted with the same buffer. A control without dye was run under the same conditions. The concentrations of bis(Ans) and DNA were monitored by the absorption at 387 and 260 nm, respectively.

Glycerol Gradient Zone Centrifugation. The glycerol gradient centrifugation studies on the aggregation states of RNA polymerase were conducted with the Beckman Spinco Model L65 ultracentrifuge using an SW 41 rotor speed of 50 000 rpm for 20 h. The buffers used were either the low-salt (0.05 M KCl) or high-salt (0.5 M KCl) solutions containing 0.05 M Tris-HCl (pH 8), 0.1 mM EDTA, 0.1 mM dithiothreitol, 0.01 M $MgCl_2$, and 5% glycerol. For the reaction of enzyme with dye, enzyme (1.3×10^{-6} M) was preincubated at 37 °C for 10 min with various concentrations of bis(Ans) in the high-salt buffer. The enzyme–dye mixture was then layered on a 13-mL, 10–30% glycerol gradient in the high-salt buffer containing the same concentration of dye used in the preincubation mixture. After centrifugation, the gradient was collected into 0.5-mL fractions. The tryptophan (340 nm) and bis(Ans) (540 nm) fluorescence intensities of each fraction were measured.

Spectrophotometric Measurements. Fluorescence spectra were recorded using a Hitachi Perkin-Elmer fluorescence spectrophotomer, Model MPF-3, equipped with a corrected spectra accessory. The cell compartment was thermostatically controlled at 22 ± 0.1 °C for all measurements. For fluorescence titrations, a 5-μL aliquot of dye or enzyme solution was added to a cuvette which contained 0.5 mL of reaction mixture. The excitation and emission wavelengths of bis(Ans) used for the titrations were 380 and 500 nm, respectively. The buffer used in fluorescence measurements was 0.05 M Tris-HCl (pH 8), 0.2 M KCl, 0.1 mM dithiothreitol, and 0.1 mM EDTA (buffer A).

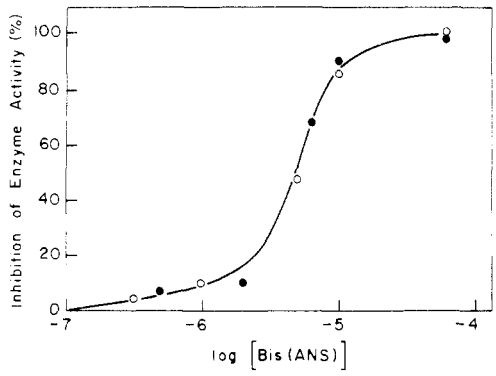

FIGURE 2: Effect of the bis(Ans) concentration on RNA synthesis at 37 °C. The reaction mixture and conditions of RNA polymerase assay were as described in the Experimental Section. Bis(Ans) was added immediately before the reaction was started. Different templates were employed: (●) calf-thymus DNA; (○) T7 DNA.

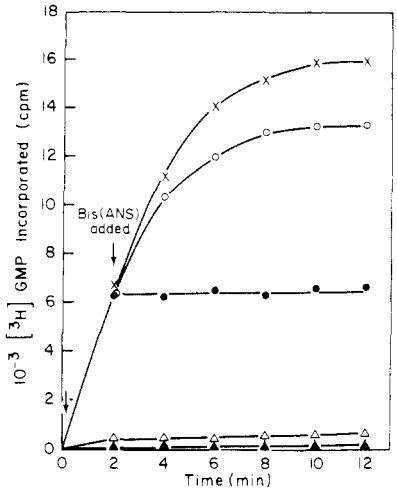

FIGURE 3: Time course of inhibition of RNA polymerase reaction: addition of bis(Ans) at various times. The reaction mixture and conditions of the polymerization assay were as described in the Experimental Section with calf-thymus DNA as template: (×) no bis(Ans) added; (▲) 10^{-4} M bis(Ans) added at 0 min; (△) 10^{-5} M bis(Ans) added at 0 min; (●) 10^{-4} M bis(Ans) added at 2 min; (○) 10^{-5} M bis(Ans) added at 2 min after the reaction started.

Results

Inhibition of RNA Synthesis by Bis(Ans). Figure 2 shows RNA synthesis in the presence of various concentrations of bis(Ans). About 90% of the RNA polymerase activity is inhibited by 1×10^{-5} M bis(Ans). At the same concentration, no inhibition was observed with Ans. The concentration of bis(Ans) required for 50% inhibition is about 4×10^{-6} M using either calf-thymus or T7 DNA as template. Preincubation of the enzyme with DNA and/or nucleoside triphosphates (Table I) will give partial protection against the inhibition. The protection effect is greater with DNA (30–50%) than with nucleoside triphosphate (12–16%), and both effects are approximately additive.

The effect of bis(Ans) addition at various times during the RNA polymerase reaction is shown in Figure 3. When 10^{-5} or 10^{-4} M bis(Ans) was added together with the enzyme to start the reaction, complete inhibition of nucleotide incorporation into RNA chains occurred instantaneously. A similar inhibition pattern was also observed when 10^{-4} M bis(Ans) was added 2 min after the reaction was started. In contrast, when 10^{-5} M bis(Ans) was added at 2 min, gradual and partial inhibition was observed. These observations indicate that RNA chain elongation is less sensitive to the dye inhibition than is chain initiation.

TABLE I: Effect of DNA and NTP on the Inhibition of RNA Polymerase by Bis(Ans).[a]

Preincubation mixture	% act. at preincubation temp (°C) 37	0
E	2.8	3.3
E + ATP + CTP + UTP + GTP	15.5	11.6
E + calf-thymus DNA	49.1	31.2
E + calf-thymus DNA + ATP + CTP + UTP	69.9	30.4

[a] The assay mixture for enzyme activity (0.25 mL) was as described in the Experimental Section. The preincubation mixture contained 0.08 M Tris-HCl (pH 7.8), 10 mM $MgCl_2$, 4 mM β-mercaptoethanol, 2 μg of enzyme, and, as indicated, 0.03 μmol of calf-thymus DNA and/or 0.4 mM NTP. After preincubation for 10 min at 37 or 0 °C, the remaining components of the assay mixture and 10^{-5} M bis(Ans) were added, and the incubation was continued for 10 min at 37 °C. 100% activity represents the activity of enzyme in the absence of bis(Ans) and is equivalent to 1.5 nmol of [^{3}H]GMP incorporated.

TABLE II: Effect of Bis(Ans) on the T7 DNA-Directed Incorporation of [γ-^{32}P]ATP, [γ-^{32}P]GTP, and [^{3}H]UMP into RNA Chains.[a]

[Bis(Ans)] (M)	Nucleotide incorp. [γ-^{32}P]-ATP (pmol)	[γ-^{32}P]-GTP (pmol)	[^{3}H]-UMP (nmol)	Av chain length, 4[^{3}H]UMP/([γ-^{32}P]ATP + [γ-^{32}P]GTP)
0	4.44	2.26	6.13	3659
1×10^{-6}	3.98	1.64	5.49	3866
5×10^{-6}	2.17	1.39	3.28	3685
1×10^{-5}	0.66	0.20	0.83	3860
1×10^{-4}	0	0	0	

[a] [γ-^{32}P]ATP or [γ-^{32}P]GTP incorporation was assayed as follows: reaction mixtures (0.1 mL) contained 50 mM Tris-HCl (pH 7.8), 10 mM β-mercaptoethanol, 10 mM $MgCl_2$, 0.4 mM each of CTP, GTP (or ATP), and UTP, 0.1 mM [γ-^{32}P]ATP (or GTP) (~4 × 10^3 cpm/pmol), 10 nmol of T7 DNA, 10 μg of enzyme, and bis(Ans) as indicated. The incubation was for 20 min at 37 °C. The reaction mixtures were then chilled, and 0.1 mL of 0.1 M sodium pyrophosphate, 0.02 mL of 50 mM ATP (or GTP), and 1 mg of bovine serum albumin were added. After mixing, 1 mL of 10% trichloroacetic acid was added. On standing for 10 min at 0 °C, the mixture was then centrifuged for 10 min at 10 000 rpm, and pellets were dissolved in 0.2 mL of 0.2 N ammonium hydroxide, 0.1 mL of 0.1 M sodium pyrophosphate, and 0.02 mL of 50 mM ATP (or GTP). In each solution, 2 mL of 10% trichloroacetic acid was again added and the precipitate was collected by centrifugation. The washing and precipitation procedure was repeated once. The final acid-insoluble RNA product was collected by filtration on Millipore membrane. The filter was washed with 5% trichloroacetic acid and dried, and radioactivity was determined by liquid scintillation counting. [^{3}H]UMP incorporation was assayed as described in the Experimental Section except that the ATP concentration was 0.1 mM.

Effect of Bis(Ans) on Association, Initiation, and Elongation of RNA Chains. The binding of RNA polymerase to DNA (association) was measured by the retention of the enzyme–DNA complex on a nitrocellulose filter. Essentially no effect was observed up to 10^{-5} M of dye (data not shown), at which the inhibition of RNA polymerase activity is about 90% (Figure 2). At 10^{-4} M bis(Ans), however, little or no DNA retention was detected in filter assay.

Initiation of RNA chains by RNA polymerase can be studied by measuring the incorporation of γ-^{32}P-labeled nu-

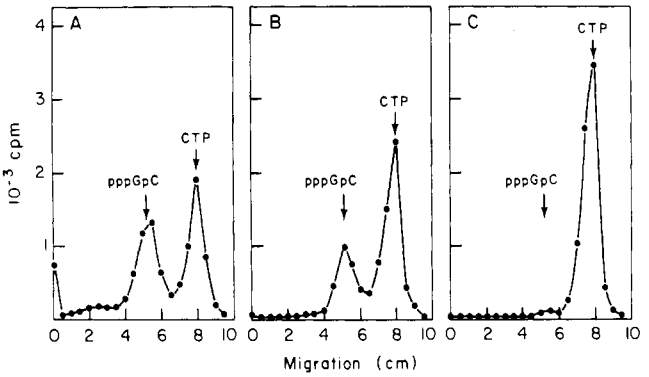

FIGURE 4: Effect of bis(Ans) and rifampicin on the T7 DNA-dependent synthesis of the dinucleoside tetraphosphate (pppGpC) catalyzed by RNA polymerase. Analysis of the reaction mixtures by paper chromatography was as described in the Experimental Section. For experiments with bis(Ans), the enzyme was preincubated with the inhibitor for 10 min at 37 °C before the reaction started: (A) no inhibitor; (B) enzyme preincubated with 6×10^{-5} M rifampicin; (C) enzyme preincubated with 7×10^{-6} M bis(Ans).

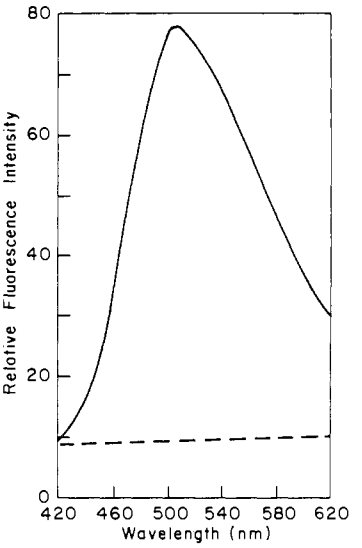

FIGURE 5: Fluorescence emission spectra of free (---) and enzyme-bound (—) bis(Ans). The solution contained 1×10^{-7} M bis(Ans) in buffer A. In the case where bound bis(Ans) was measured, 2×10^{-6} M RNA polymerase was present. The excitation wavelength was 380 nm.

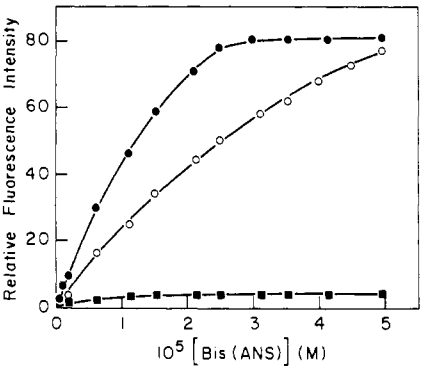

FIGURE 6: Fluorimetric titration of RNA polymerase with varying concentrations of bis(Ans) in the presence and absence of calf-thymus DNA. The concentrations for enzyme and DNA were 10^{-6} M and 0.5 mg/mL, respectively. The excitation wavelength was 380 nm and the emission wavelength was 500 nm: (●) RNA polymerase alone; (■) calf-thymus DNA alone; (○) RNA polymerase and calf-thymus DNA preincubated for 10 min at 37 °C.

cleoside triphosphates into RNA (Maitra and Hurwitz, 1965), since the initial nucleotide of a de novo synthesized RNA chain retains the β- and γ-phosphate groups during subsequent chain elongation. The ratio of ^{3}H-labeled nucleoside monophosphate to γ-^{32}P-labeled nucleoside triphosphate incorporated is thus a measure of the average chain length of RNA synthesized in the polymerase reaction. The effects of bis(Ans) on the initiation and elongation of RNA chains using T7 DNA as template are given in Table II. Lower concentrations of bis(Ans) ($\leq 10^{-5}$ M) preferentially inhibited chain initiation. There was little effect on chain elongation as shown by the unaltered average chain length. At higher concentrations of bis(Ans) (10^{-4} M), both [γ-^{32}P]NTP and [^{3}H]NMP incorporations were completely inhibited. This was probably due to the inhibitions of the enzyme-template association under these conditions.

Effect of Bis(Ans) on DNA-Dependent Dinucleoside Tetraphosphate Synthesis. It has recently been shown that RNA polymerase catalyzes a DNA-dependent synthesis of dinucleoside tetraphosphate (Johnston and McClure, 1976). This synthesis occurs when the promoter-bound RNA polymerase is incubated with the first two nucleotides specified by the initiating DNA sequence. Initiation at the A_2 promoter on T7 DNA results in a RNA product beginning with the sequence GCU . . . (Pribnow, 1975). Incubation of this DNA with RNA polymerase, GTP, and CTP results in the formation of pppGpC (H. Oen, unpublished data). We have found that 7×10^{-6} M bis(Ans) completely inhibits this synthesis. This is shown in Figure 4. Under similar conditions, 6×10^{-5} M rifampicin only partially inhibits this reaction, consistent with the results of Johnston and McClure (1976).

Binding of Bis(Ans) to RNA Polymerase and Calf-Thymus DNA. Whether bis(Ans) is bound to RNA polymerase or to DNA was determined by gel filtration. When a mixture of bis(Ans) and RNA polymerase (or a mixture of the dye and calf-thymus DNA) was passed through a Sephadex G-75 column, the high molecular weight enzyme (or DNA) was totally excluded from the gel and appeared in the void volume. It was observed that a bis(Ans) peak coincided with the enzyme peak but not with the DNA peak. The free dye appeared later in the elution. These results demonstrate that bis(Ans) binds to the enzyme but not to DNA.

The binding of bis(Ans) to RNA polymerase is further confirmed by fluorescence spectroscopy. Figure 5 shows the fluorescence emission spectra of free and enzyme-bound bis(Ans). On excitation at 380 nm, free bis(Ans) in aqueous buffer solution was virtually nonfluorescent. Addition of RNA polymerase to this solution produced a marked enhancement of fluorescence intensity with an emission maximum at 500 nm. By monitoring the fluorescence change at 500 nm, we have performed the fluorimetric titration of RNA polymerase with varying concentrations of bis(Ans) (Figure 6). From the titration curve, the concentration of bis(Ans) required to obtain half of the maximal fluorescence enhancement is about 8×10^{-6} M. If the enzyme was preincubated with calf-thymus DNA and then titrated with bis(Ans), the concentration of dye required to obtain half-maximal fluorescence enhancement was higher than for enzyme alone. No fluorescence change was seen when calf-thymus DNA was titrated with the dye in the absence of enzyme.

Quantitative Analysis of the Dye-Enzyme Interaction. Quantitative analysis of the stoichiometry and equilibrium parameters of the dye-enzyme interaction was performed by fluorimetric titration of bis(Ans) with varying concentrations of RNA polymerase. From the titration data obtained, one can

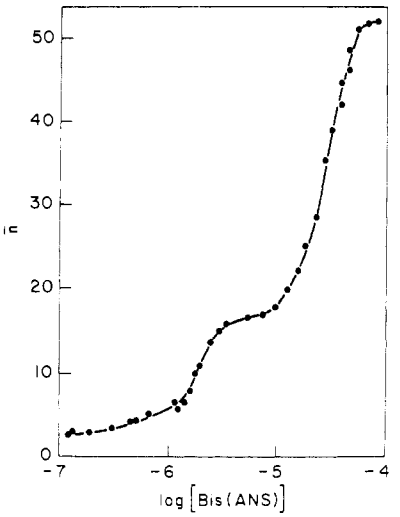

FIGURE 7: The binding isotherm for the interaction of bis(Ans) with RNA polymerase obtained by fluorimetric titration. $\bar{n}$ is the average number of bis(Ans) molecules bound per enzyme molecule. [Bis(Ans)] is the free dye concentration. The bis(Ans) concentration was fixed at 10^{-4}, 10^{-5}, or 10^{-6} M. The enzyme concentration was varied from 2×10^{-7} to 5×10^{-6} M in each titration. The excitation and emission wavelengths were 380 and 500 nm, respectively.

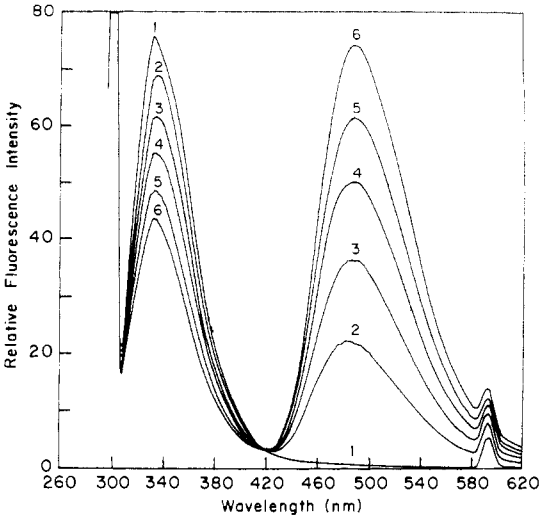

FIGURE 8: Quenching of tryptophan fluorescence of RNA polymerase by bis(Ans). The excitation wavelength was 296 nm. The concentration of enzyme was 4×10^{-7} M. The concentration of bis(Ans) was 0, 2, 4, 6, 8, and 10×10^{-6} M for curves 1 to 6, respectively. The peaks near 330 nm represent tryptophan fluorescence of the enzyme, while those at 490 nm represent bis(Ans) fluorescence. The small peaks at 592 nm are due to the second-order Rayleigh scattering.

calculate $\bar{n}$, the average number of moles of dye bound per mole of enzyme using the equation $\bar{n} = x[L_0]/[E_0]$, where $[L_0]$ and $[E_0]$ represent the total dye and enzyme concentrations, respectively, and x is the fraction of dye molecules bound. Assuming that the quantum yield of all the bound dye is the same, $x = F/F_b$, where F is the fluorescence intensity at a certain value of x and F_b is the fluorescence intensity when all dye molecules are bound. The plot of $\bar{n}$ vs. log [L], where [L] is the free dye concentration, is shown in Figure 7. According to the method of Weber and Anderson (1965), one can estimate the total number of binding sites of bis(Ans) on RNA polymerase to be about 52 from the binding isotherm. Furthermore, there are two distinct classes of binding sites: a class of 16–18 strong binding sites with apparent $K_d = 2 \times 10^{-6}$ M, and a class of 34–36 weak binding sites with apparent $K_d \simeq 2 \times 10^{-5}$ M. The binding of bis(Ans) to both strong and weak binding sites exhibits positive cooperativity as marked by the relatively steep rising of $\bar{n}$ at the concentrations near the values of two apparent K_ds.

Quenching of Tryptophan Fluorescence of RNA Polymerase by Bis(Ans). Upon excitation at 296 nm, RNA polymerase fluoresces with a maximum near 330 nm due to tryptophan emission. This is shown in Figure 8. When increasing concentrations of bis(Ans) were added to the enzyme solution, the bis(Ans) fluorescence (490 nm) increases with a concurrent decrease in the tryptophan fluorescence (330 nm), resulting in an isoemissive point at 420 nm. At 10^{-5} M bis(Ans), quenching of tryptophan fluorescence was about 40–50%.

Effect of Bis(Ans) on the Aggregation State of RNA Polymerase. The aggregation state of RNA polymerase was studied by 10–30% glycerol gradient zone centrifugation. It is known (Berg and Chamberlin, 1970) that RNA polymerase holoenzyme is a monomer in high-salt (0.5 M KCl) buffer and dimerizes in low-salt (0.05 M KCl) buffer. This is demonstrated by the control experiments in Figure 9A. Figure 9B shows the aggregation state of RNA polymerase in high-salt buffer in the presence of four different concentrations of bis(Ans). When the concentration of bis(Ans) was $<10^{-5}$ M, the enzyme sedimented primarily as a monomer though there appeared to be a small amount of slower sedimenting material as well. At dye concentration $>10^{-5}$ M, the enzyme formed mostly dimers and some higher aggregates dissociated into monomer when they were recentrifuged in the same gradient without bis(Ans), indicating that the bis(Ans)-induced enzyme aggregation is reversible.

Discussion

The present study demonstrates that bis(Ans) inhibits bacterial DNA-dependent RNA synthesis by complexing with RNA polymerase. There are multiple bis(Ans) binding sites on the enzyme: 16–18 strong sites with an apparent K_d of $\sim 10^{-6}$ M and 34–36 weak sites with an apparent K_d of $\sim 10^{-5}$ M. The large number of bis(Ans) binding sites (a total of 52) on RNA polymerase is not surprising. It was reported (Anderson, 1971) that lactic dehydrogenase, whose molecular weight is about $\frac{1}{4}$–$\frac{1}{3}$ that of RNA polymerase, contains 10–12 primary binding sites for bis(Ans).

Since bis(Ans) is an aromatic compound, one would expect that it would interact with aromatic amino acid residues in the enzyme through hydrophobic interactions. This is, in fact, the case as shown by the quenching of tryptophan fluorescence of RNA polymerase by bis(Ans) (Figure 8). There are 25 tryptophan residues in *E. coli* RNA polymerase (Fujiki and Zurek, 1975), about 50% of which are exposed (Nicholson, 1971). These residues on the surface of the enzyme are most likely to be bound by bis(Ans). Upon excitation at 296 nm, about 50% of the tryptophan fluorescence is quenched by 10^{-5} M bis(Ans). The present data, however, are not sufficient to permit calculation of the number of tryptophan residues involved in the dye binding. It is interesting to note that binding of nucleoside triphosphate also quenches the tryptophan fluorescence of RNA polymerase (Wu and Goldthwait, 1969). Thus, it is probable that bis(Ans) would interact with some aromatic acid residues that are near or at the NTP binding sites on the enzyme. The facts that the aromatic region of bis(Ans) is twice as large as that of Ans and hence may have much stronger affinity for the hydrophobic sites on the enzyme may explain why bis(Ans) is a much more potent inhibitor than Ans.

At lower concentrations ($\leq 10^{-5}$ M), bis(Ans) selectively

inhibits RNA chain initiation but not chain elongation. Similar selectivity was shown for the inhibition by rifampicin of RNA synthesis (Hartmann et al., 1967). However, unlike bis(Ans), rifampicin exerts its effect by binding to a single site on RNA polymerase (di Mauro et al., 1969; Wehrli and Staehelin, 1969), although there are indications that another weak binding site may exist (Riva et al., 1972; Fietta and Sylvestri, 1975). From the fluorimetric titration studies (Figure 7), we have shown that at 10^{-5} M, bis(Ans) binds to approximately 16–18 strong sites. Whether the binding to some or all of these primary sites is responsible for inhibition is not known. Nevertheless, there is rough agreement between the inhibition constant and the apparent K_d obtained by fluorimetric titration (Figure 7). Also, the shapes of the inhibition curve (Figure 2) and the binding isotherm (Figure 7) resemble each other in the concentration range from 10^{-6} to 10^{-5} M. These results suggest that binding to some or all of the strong sites may be responsible for inhibition. In addition, both DNA and nucleoside triphosphates partly protect the enzyme from inhibition by bis(Ans) (Table I). This protection effect appears to be a result of the template- or substrate-induced conformational change of the enzyme that renders some of the inhibitor binding sites less accessible. This was shown in the case of DNA by fluorimetric titrations in the presence and absence of DNA (Figure 6).

The detailed mechanism by which bis(Ans) inhibits the initiation of RNA synthesis remains to be elucidated. Inhibition of RNA chain initiation could be attributed to the inhibition of one or more of the following steps: the association of the enzyme with DNA template, the binding of the substrates to the binary complex, the formation of the first phosphodiester bond, or the translocation assumed to be necessary before the second bond can be formed. At the concentrations of bis(Ans) that selectively inhibit initiation, the association of DNA-enzyme complex is not altered. Moreover, the observation that bis(Ans) inhibits the DNA-dependent dinucleotide synthesis indicates that the dye blocks either the substrate binding or the subsequent phosphodiester bond formation. Alternatively, bis(Ans) could inhibit the release of the dinucleotide from the enzyme and template in this abortive initiation system. None of these steps are affected by rifampicin (Johnston and McClure, 1976).

At higher concentrations of bis(Ans) ($\geq 10^{-4}$ M), the enzyme forms dimers and higher aggregates which are unable to bind DNA. The aggregation of RNA polymerase induced by bis(Ans) is reversible. It has been shown (Anderson, 1971) that bis(Ans) converts beef lactic dehydrogenase from monomer (7 S) to tetramer (22 S), which can be reversed by addition of NADH (Lu and Anderson, 1973). There are two possible mechanisms by which bis(Ans) may induce dimerization or aggregation of an oligomeric enzyme. First, bis(Ans) could act like a cross-linker to associate two or more enzyme protomers through hydrophobic interaction of the dye with enzyme. This cannot be a general mechanism since bis(Ans) was found to cause the dissociation of aspartate transcarbamoylase (G. Weber, personal communication). The second possibility is that bis(Ans) binds to the hydrophobic region of enzyme which is involved in the protomer-protomer interactions. Thus, depending on the nature of such interactions, the dye binding could either stabilize or destabilize the contact domain between protomers. This could explain the small amount of dissociation into subunits we observed on our gradients (Figure 9B). In this connection, Krakow and von der Helm (1970) have reported that Congo red, a dye with a structure very similar to that first proposed for bis(Ans) (Rosen and Weber, 1969), inhibits the binding of RNA polymerase to DNA and induces the dissociation of the enzyme into its subunits.

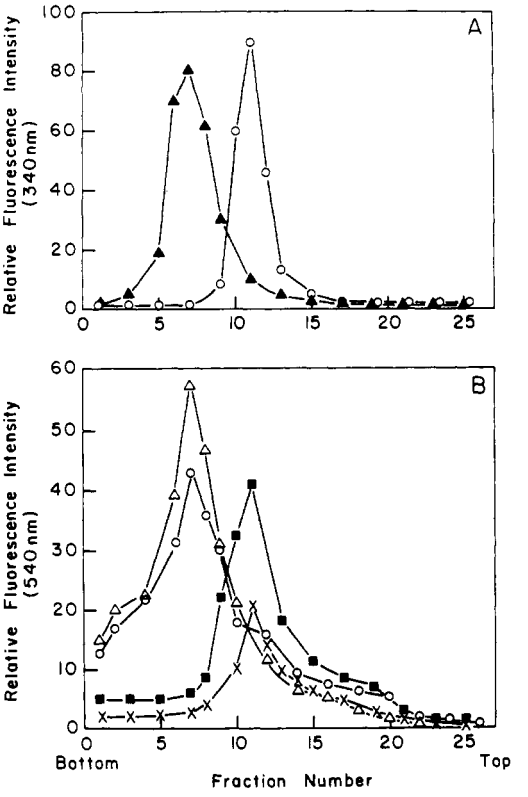

FIGURE 9: Glycerol-gradient centrifugation studies of RNA polymerase (A) and RNA polymerase-bis(Ans) complex (B). The reaction mixture and conditions used were as described in the Experimental Section. The enzyme concentration was 1.3×10^{-6} M. (A) (○) Enzyme monomer in the high-salt (0.5 M KCl) buffer; (▲) enzyme dimer in the low-salt (0.05 M KCl) buffer. The presence of enzyme in each fraction was determined by tryptophan fluorescence at 340 nm. The excitation wavelength was 296 nm. (B) The bis(Ans) concentration was 10^{-4} M (△), 5×10^{-5} M (○), 10^{-5} M (■), and 4×10^{-6} M (×) in the high-salt buffer. The presence of the enzyme-dye complex was determined by the dye emission at 540 nm. The excitation wavelength was 430 nm.

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Lysine Transfer RNA_2 Is the Major Target for L-Ethionine in the Rat†

Yoshiyuki Kuchino,‡ Opendra K. Sharma, and Ernest Borek*

ABSTRACT: Ethionine, a hepatocarcinogen, ethylates macromolecules in vivo especially tRNA of rat liver. When rats were injected with L-[*ethyl*-^{3}H]ethionine, the tRNA fraction of the liver was found to be labeled. One tRNA with the highest specific activity was purified and identified as lysine-$tRNA_2$.

It is well known that chemical carcinogens are highly reactive with some cellular macromolecules. Magee and Farber showed that tRNAs have a greater propensity for alkylation by some carcinogens than DNA (Farber and Magee, 1960; Magee and Farber, 1962). Whether the modification of tRNAs by chemical carcinogens has any relation to oncogenesis is obscure at present. However, all tumors of a wide spectrum of etiology contain a small number of isoaccepting tRNAs which are absent from the tissue of origin (Borek and Kerr, 1972). One of these tRNAs, $tRNA^{Phe}$, has been isolated from two different neoplastic tissues and each was found to contain three supernumerary modified bases (Kuchino and Borek, 1977). The interaction of the macromolecules with chemical carcinogens may be an initiating event in carcinogenesis.

Ethionine is a hepatocarcinogen in rats. Feeding of ethionine to rats results in the loss of two leucine tRNA species in the liver (Axel et al., 1967). These changes are evident much before the appearance of tumors. However, the two leucine tRNA species reappear (Yamane et al., 1976) on supplementation of the diet with copper, an inhibitor of ethionine carcinogenesis (Kamamoto et al., 1973). Ethionine interacts preferentially with liver tRNA (Farber and Magee, 1960; Farber et al., 1967; Ortwerth and Novelli, 1969), resulting predominantly in the formation of ethylated guanosines (Rosen, 1968; Pegg, 1972). However, whether the targets of the ethylation are random or specific in the population of tRNAs was obscure. We present here evidence that $tRNA^{Lys}_2$ is a specific target of alkylation by ethionine.

Materials and Methods

L-[*ethyl*-^{3}H]Ethionine (spec act. 70.1 Ci/mol) was purchased from New England Nuclear, Boston, Mass., ^{14}C- and ^{3}H-labeled L-amino acids were obtained from Amersham/Searle Corp., Arlington Heights, Ill., and RNase T_1, RNase A, RNase T_2, and bacterial alkaline phosphatase were products of Worthington Biochemical Co., St. Louis, Mo., Sigma Chemical Co., St. Louis, Mo., and Sankyo Co. Ltd., Tokyo, Japan.

Preparation of tRNA from Rat Liver after Exposure to L-Ethionine. Three female Holtzman rats (weighing 140 to 160 g) fasted overnight were injected intraperitoneally with 0.5 mCi of L-[*ethyl*-^{3}H]ethionine in 0.9% NaCl solution. After starvation for 24 h, the rats were sacrificed and the livers were rapidly removed. The tRNA was prepared by the methods described previously (Brunngraber, 1962; Zubay, 1962) and deacylated by incubation with 1.8 M Tris-HCl (pH 8.0) at 37

† From the Department of Microbiology, University of Colorado Medical Center, Denver, Colorado 80262. *Received July 26, 1977.* This work was supported by Research Grant CA 14047-05 from the National Cancer Institute.

‡ Present address: Biology Division, National Cancer Center Research Institute, Tsukiji-5-1-1 Chuo-Ku, Tokyo, Japan.

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