Rose Bengal: an Inhibitor of Ribonucleic Acid Chain Elongation†

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ABSTRACT: Rose Bengal is a potent inhibitor of the DNA-dependent RNA polymerase of *Escherichia coli* and photooxidation is not necessary for this inhibition. Virtually no inhibition occurs at 10^{-7} M Rose Bengal, while complete inhibition is reached at about 10^{-5} M. The concentration of Rose Bengal required to inhibit 50% of RNA polymerase activity is 1.4×10^{-6} M and is independent of the DNA or synthetic polynucleotides used as template. Kinetic analysis reveals a noncompetitive inhibition of Rose Bengal with respect to nucleoside triphosphates. This suggest that the inhibitor does not affect the combination of the substrate with the enzyme. Similarly, Rose Bengal ($\leq 10^{-5}$ M) does not alter the binding of RNA polymerase to T7 DNA as measured by a nitrocellulose filter assay. At lower concentrations (10^{-6} M), Rose Bengal selectively inhibits RNA chain elongation compared to initiation (This was demonstrated by the differential effects of Rose Bengal on [^{3}H]UMP *vs.* [γ-^{32}P]ATP incorporation into RNA chains.), resulting in the formation of smaller RNA products. Rose Bengal also exhibits a selective inhibition of the DNA-dependent [^{32}P]PP$_i$-exchange reaction catalyzed by RNA polymerase. With d(A-T) copolymer, the exchange of [^{32}P]PP$_i$ with UTP and ATP was inhibited by 10^{-6} M Rose Bengal, while this concentration had no effect on the [^{32}P]PP$_i$ exchange when ATP was replaced by AMP, ADP, or UpA. It is suggested that Rose Bengal might either inhibit the formation of phosphodiester bonds or block the translocation of enzyme along the template. At higher concentrations (10^{-5} M), Rose Bengal also inhibits chain initiation and contributes to the premature release of nascent RNA chains from the enzyme–DNA complex. Furthermore, evidence is presented that Rose Bengal binds reversibly to RNA polymerase but does not bind to DNA.

The synthesis of RNA by DNA-dependent RNA polymerase is a complex process and can be divided into several successive steps (Goldthwait *et al.*, 1970): (a) the binding of the enzyme to the DNA template (association), (b) the binding of ribonucleoside triphosphates (NTP)[1] to the enzyme–DNA complex followed by the formation of the first phosphodiester bond (initiation). (c) the subsequent addition of NTP to form a polyribonucleotide chain (elongation). and (d) the cessation of chain growth with the release of the RNA product from the enzyme and the template (termination). Several inhibitors are known to inhibit specifically one or more of these steps. For example, rifamycins inhibit initiation whereas streptolydigin affects association, initiation, as well as elongation. These inhibitors are important tools for the investigation of gene transcription.

Rose Bengal has been used as a probe for active sites of enzymes by photosensitized oxidation (Westhead, 1965; Hoffee *et al.*, 1967). Ishihama and Hurwitz (1969) have studied photooxidation of RNA polymerase in the presence of Rose Bengal. They found that the photooxidized enzyme was unable to elongate RNA chains but still retained the ability to bind DNA and ribonucleoside triphosphates, and to catalyze a rapid DNA-dependent [^{32}P]PP$_i$ exchange reaction.

Since Rose Bengal is fluorescent, the possibility of using Rose Bengal as a spectroscopic probe for studies with RNA polymerase necessitated a more detailed study of the mode of action of this reagent. The results of such a study, presented in this paper, show that Rose Bengal is a potent inhibitor of RNA polymerase even in the absence of photooxidation. At low concentrations (10^{-6} M) it preferentially inhibits chain elongation. However, at higher concentrations (10^{-5} M) it also inhibits initiation and facilitates the release of nascent RNA chains from the enzyme–DNA complex. It is suggested that Rose Bengal might either inhibit the formation of phosphodiester bonds or block the translocation of enzyme along the template. Furthermore, Rose Bengal binds only to the enzyme and not to DNA. A spectroscopic study of Rose Bengal–RNA polymerase interaction is presented in the subsequent paper.

Experimental Section

Reagents. Unlabeled ribonucleoside triphosphates were purchased from P-L Biochemicals. ^{3}H-Labeled ribonucleoside triphosphates and ^{32}P-labeled sodium pyrophosphate were obtained from New England Nuclear Corp. Poly[d-(A-T)] and calf thymus DNA were products of Miles Laboratories, Inc., and Worthington Biochemical Corp., respectively. [^{3}H]T7 DNA was a generous gift from Dr. L. Yarbrough, and [γ-^{32}P]ATP was a gift from Dr. U. Maitra. Rose Bengal was purchased from Fisher Scientific Co. and further purified by Bio-Rad AG 50-X8 cation-exchange chromatography (Hoffee *et al.*, 1967).

Reagents for the preparation of RNA polymerase were obtained from the following sources: *Escherichia coli* B (mid log, high peptone medium), General Biochemicals, Polyethylene Glycol 6000, J. J. Baker Chemical Co.; Dextran T-500, Pharmacia Fine Chemicals, Inc.; agarose, Sigma Chemical Co.; Agarose 1.5M and 5M, Bio-Rad Laboratories. DNA–agarose was prepared according to the procedure of Schaller *et al.* (1972).

RNA Polymerase. RNA polymerase was prepared from *E.*

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[1] Abbreviations used are: NTP, nucleoside triphosphate; PP$_i$, inorganic pyrophosphate.

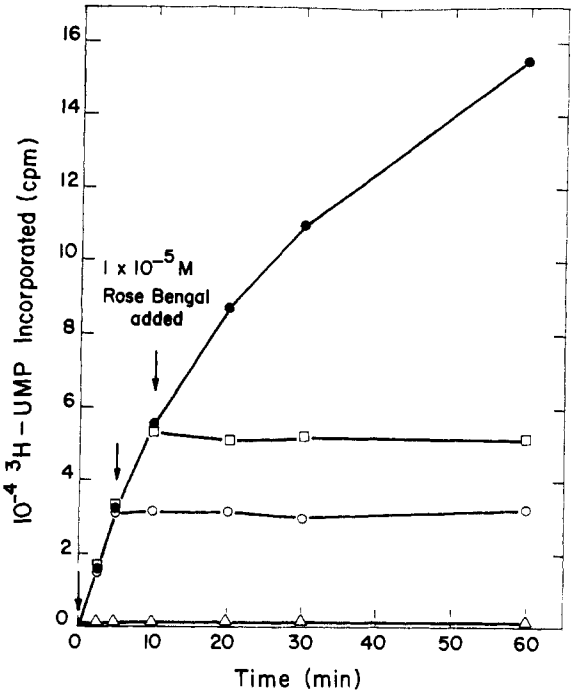

FIGURE 1: Rose Bengal inhibition of RNA polymerase reaction. The reaction mixture and conditions of the polymerization assay were as described in the Experimental Section with poly[d(A-T)] as template and 10^{-5} M Rose Bengal. RNA polymerase was added to start the reaction and incubation was in the dark. (●) No Rose Bengal added; (△) Rose Bengal added at 0 min; (○) 5 min; and (□) 10 min after the reaction was started.

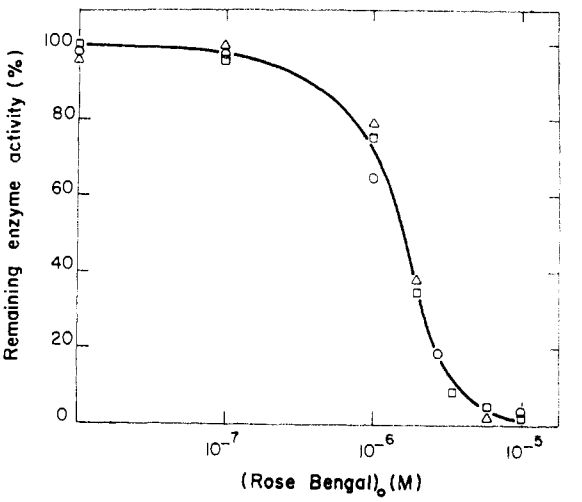

FIGURE 2: The dependence of RNA synthesis on the concentration of Rose Bengal. The enzyme activity remaining (per cent) after exposure to Rose Bengal was plotted against the concentration of Rose Bengal added. The reaction mixture and conditions of the polymerization assay were as described in the Experimental Section. Rose Bengal was added immediately before the reaction was started. Incubation was at 37° in the dark. Different templates were employed: (□) calf thymus DNA; (○) T7 DNA; (△) poly[d(A-T)].

coli cells by modification of the method of Yarbrough and Hurwitz (1973). *E. coli* cells (400 g to 1 kg) were disrupted in a Waring Blendor or by passage through the Manton-Gaulin laboratory homogenizer. After centrifugation, the crude extract was treated with polyethylene glycol and dextran. The polyethylene glycol phase was dialyzed and passed through a DNA-agarose column. The column was washed with 1.2 M NaCl buffer and the fractions of high enzyme activity were combined. The enzyme was further purified by chromatography on an agarose (1.5M) column in the presence of high salt (1 M NaCl), followed by chromatography on an agarose (5M) column in the presence of low salt (0.05 M NaCl). The purity of the enzyme thus obtained was greater than 98% based on sodium dodecyl sulfate gel electrophoresis (Burgess, 1969). The purified enzyme was stored at −20° in the storage buffer (60% glycerol, 0.2 M KCl, 0.05 M Tris-HCl (pH 8), 0.1 mM EDTA, and 1 mM dithiothreitol) and was stable for several months.

Polymerization Assay. RNA polymerase activity was assayed by the incorporation of ^{3}H-labeled ribonucleoside monophosphate into acid-insoluble material as described previously (Wu and Goldthwait, 1969). 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, GTP, CTP, and UTP (one labeled with ^{3}H, 5×10^3 cpm/nmol), 1.6 mM sodium phosphate, 0.03 μmol calf thymus DNA (or T7 DNA), and 2–5 μg of the enzyme. When poly[d(A-T)] was used as a template, GTP and CTP were omitted and 0.2 M KCl was added to the reaction mixture. The incubation was for 10 min at 37° in the dark. At the end of the incubation the reaction mixture was chilled in 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.

Other Assay Methods. 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). The initiation of RNA chains was measured by the incorporation of [γ-^{32}P]ATP into the acid-insoluble material as described by Maitra *et al.* (1967) with slight modification. The procedure used for measuring the DNA-dependent [^{32}P]PP_i-exchange catalyzed by RNA polymerase was essentially as described by Krakow and Fronk (1969). The release of RNA chains from the DNA-enzyme complex was determined by the retention of [^{3}H]RNA on a nitrocellulose filter as carried out by Richardson (1969).

All the reactions involving Rose Bengal were carried out in the dark to eliminate the effects of light. The transfer and addition of Rose Bengal were done in dim light.

Results

Inhibition of RNA Synthesis by Rose Bengal in the Absence of Photooxidation. When experiments were carried out in the dark to ensure no photosensitized oxidation, nucleotide incorporation into RNA chains catalyzed by RNA polymerase was inhibited almost instantaneously by addition of Rose Bengal (10^{-5} M) at any time during the reaction (Figure 1). RNA synthesis in the presence of various concentrations of Rose Bengal was studied (Figure 2). Virtually no inhibition occurred up to 10^{-7} M Rose Bengal, while complete inhibition was observed at about 10^{-5} M. The concentration of Rose Bengal required for 50% inhibition was about 1.4×10^{-6} M. This value was independent of DNA or synthetic polynucleotides used as template. Moreover, replacement of Mg^{2+} by Mn^{2+} did not alter the quantitative effects of Rose Bengal.

Influence of Nucleotide Concentration on Rose Bengal Inhibition. The effect of nucleotide concentration on Rose Bengal inhibition was studied using poly[d(A-T)] as template. At saturating concentration of either ATP or UTP (0.4 mM), variation of the alternate ribonucleoside triphosphate yielded

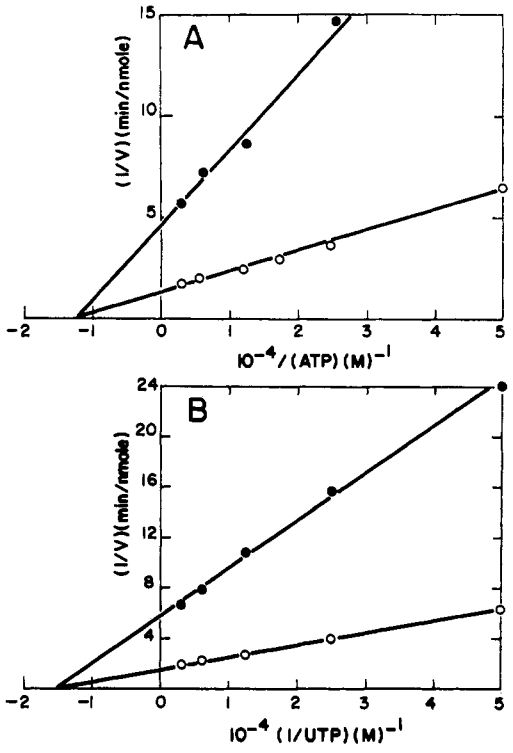

FIGURE 3: Effect of Rose Bengal on the double-reciprocal plot of AMP (A) or UMP (B) incorporation using poly[d(A-T)] as a template. The reaction mixture and conditions of polymerization assay were the same as described in the Experimental Section, except that [^{3}H]ATP (or [^{3}H]UTP) was held constant at 0.4 mM and UTP (or ATP) concentration was varied from 0.02 to 0.32 mM; (○) no Rose Bengal. (●) 1.8×10^{-6} M Rose Bengal was added immediately before the reaction was started with the enzyme.

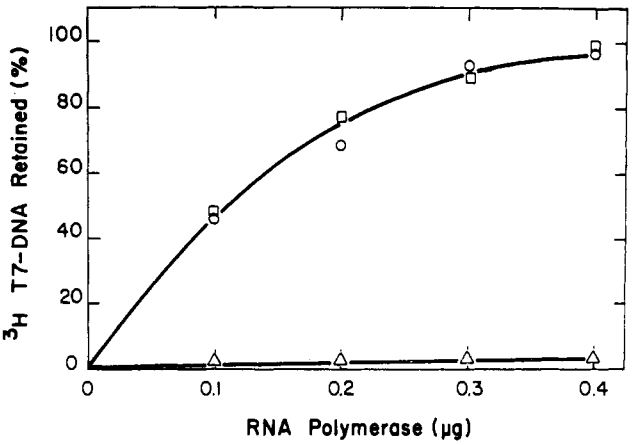

FIGURE 4: Effect of Rose Bengal on the binding of RNA polymerase to T7 DNA. The modified procedure of Hinkle and Chamberlin (1970) was used. Binding mixture (0.1 ml) contained 5 nmol of ^{3}H-labeled T7 DNA (12,500 cpm) and enzyme (amount as indicated) in the binding buffer (0.01 M Tris-HCl, pH 8, 0.01 M $MgCl_2$, 0.01 M β-mercaptoethanol, 1 mM EDTA, and 0.05 M NaCl) which also contained 0.5 mg/ml of bovine serum albumin; 0.01 ml of Rose Bengal (10^{-3} or 10^{-4} M) or H_2O (control) was then added to the binding mixture. After incubation for 5 min at 37° in the dark, the mixture was chilled in ice, diluted with 2 ml of binding buffer, and filtered through Millipore membrane with gentle suction (4–6 cm). The filter was washed with another 2 ml of the binding buffer (without bovine serum albumin), dried, and counted. (□) Control; (○) in the presence of 1×10^{-5} M Rose Bengal; (△) in the presence of 1×10^{-4} M Rose Bengal.

linear double-reciprocal plots (Figure 3A,B). As can be seen, Rose Bengal is a noncompetitive inhibitor with respect to the binding of ATP and UTP to RNA polymerase: the V_{max} decreased in the presence of Rose Bengal while the apparent K_m remained unchanged. The K_i value for Rose Bengal was 7×10^{-7} M, assuming a noncompetitive inhibition.

Effect of Rose Bengal on the Binding of RNA Polymerase to T7 DNA. The binding of RNA polymerase to DNA can be measured by retention of the complex on a nitrocellulose membrane filter (Freeman and Jones, 1967; Hinkle and Chamberlin, 1970). The effect of Rose Bengal on the binding of RNA polymerase to T7 DNA is shown in Figure 4. Essentially no effect was observed up to 10^{-5} M, at this concentration of Rose Bengal, inhibition of RNA polymerase activity is almost complete (Figure 2). However, at a Rose Bengal concentration of 1×10^{-4} M, little or no DNA retention was detected in the Millipore filter assay.

Effect of Rose Bengal on Initiation and Elongation of RNA Chains. Initiation and elongation of RNA chains in the DNA-dependent RNA polymerase reaction have been studied by measuring the incorporation of γ-^{32}P-labeled nucleoside triphosphate and ^{14}C- (or ^{3}H-)-labeled nucleoside monophosphates into RNA (Maitra *et al.*, 1967). 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 ^{14}C- (or ^{3}H-)-labeled nucleoside monophosphate to γ-^{32}P-labeled nucleoside triphosphate incorporated is a measure of the average chain length of RNA synthesized in the polymerase reaction. The effects of Rose Bengal on initiation and elongation of RNA chains are shown in Table I. When T7 DNA was used as a template, relatively low concentrations of Rose Bengal (10^{-6} M) preferentially inhibited elongation of RNA chains with little influence on chain initiation. At high concentrations of Rose Bengal (1×10^{-5} M), RNA synthesis was almost completely inhibited, whereas [γ-^{32}P]ATP incorporation was inhibited only 60%. This selective inhibition leads to a marked decrease in the average chain length of RNA formation, as represented by the ratio of [^{3}H]UMP: [γ-^{32}P]ATP incorporated in the presence of increasing concentrations of Rose Bengal. The decrease in the size of the RNA synthesized in the presence of Rose Bengal was confirmed by centrifugation in sodium dodecyl sulfate sucrose gradients containing formaldehyde (Maitra *et al.*, 1970). In the presence of 1.5×10^{-6} M Rose Bengal the RNA synthesized was smaller (10 S) in size than that synthesized in the absence of Rose Bengal (23 S).

Effect of Rose Bengal on the Phosphodiester-Bond Formation. The DNA-dependent PP_i–NTP-exchange reaction catalyzed by RNA polymerase has been used to measure the formation of a phosphodiester bond(s) (Krakow and Fronk, 1969). As shown in Table II, with d(A-T) copolymer, the exchange of [^{32}P]PP_i with ATP and UTP was inhibited (30%) by 10^{-6} M of Rose Bengal. At this concentration of Rose Bengal no inhibition of the [^{32}P]PP_i-exchange reaction was observed when ATP was replaced by AMP, ADP, or UpA. Under these conditions, only the formation of the first phosphodiester bond was possible (Krakow and Fronk, 1969; Downey and So, 1970). However, during exchange reactions conducted in the presence of both ATP and UTP, the formation of short chains of oligonucleotides is possible. Thus the effect of Rose Bengal may be different for the formation of the first phosphodiester bond than for the subsequent ones. Although Rose Bengal at concentration of 10^{-6} M had little or no effect on [^{32}P]PP_i exchange reactions, at concentrations of 10^{-5} M the exchange was inhibited more than 95%.

Effect of Rose Bengal on RNA Chain Release. Another

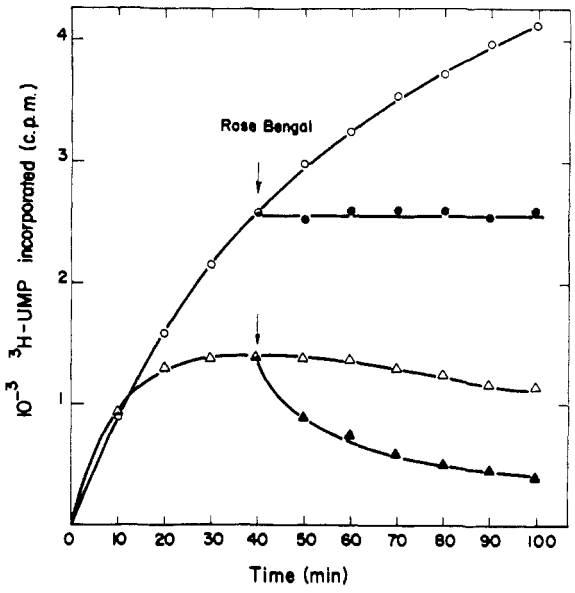

FIGURE 5: Effect of Rose Bengal on the release of RNA chains from the RNA polymerase–poly[d(A-T)] complex. The reaction mixture was the same as that used for the polymerization assay using poly[d(A-T)] as the template, except the volume was 2 ml. The incubation was at 37° in the dark and 0.25-ml aliquots were removed at various times during the incubation and assayed for the retention of [^{3}H]RNA–enzyme–DNA complex on a Millipore filter (Richardson, 1969). After 40-min incubation, the reaction mixture was divided into two portions (0.5 ml each); 0.05 ml of Rose Bengal or H_2O (control) was added immediately to each and the [^{3}H]RNA retention assay was continued. (○) Amount of RNA synthesized in the absence of Rose Bengal; (●) amount of RNA synthesized after addition of 10^{-5} M Rose Bengal; (△) retention of the RNA–enzyme–DNA complex in the absence of Rose Bengal; (▲) retention of the RNA–enzyme–DNA complex after addition of 10^{-5} M Rose Bengal.

possible explanation for the effect of Rose Bengal on RNA synthesis is that it alters the stability of the nascent DNA–enzyme–RNA complex, in such a way that RNA chains are released prematurely. This possibility was tested by measuring the retention of RNA chains by the DNA–enzyme complex on membrane filters in the presence and absence of Rose Bengal. The addition of 10^{-5} M Rose Bengal to the polymerase reaction immediately halted further RNA synthesis (Figure 1). As shown in Figure 5, RNA chains that were growing were released rapidly after addition of Rose Bengal. By 100 min only about 30% of the RNA was still retained by the filters. No significant differences were observed between the control and reaction mixtures containing 10^{-6} M Rose Bengal.

Effect of Rose Bengal on Unprimed Synthesis of Poly(rA·rU). In addition to the template-directed reactions, the unprimed synthesis of polyribonucleotide by RNA polymerase has been a subject of some interest (Krakow, 1968). Table III shows the effect of Rose Bengal on the unprimed synthesis of poly(rA·rU) catalyzed by RNA polymerase. The concentration of Rose Bengal required to inhibit unprimed synthesis was the same as that found for the inhibition of the DNA-dependent RNA polymerase reaction. Since DNA is not involved in unprimed synthesis, the results indicate that Rose Bengal inhibits the reaction through interaction with RNA polymerase.

Direct Binding of Rose Bengal to RNA Polymerase. Direct evidence that Rose Bengal binds to RNA polymerase but not to DNA was observed by means of gel filtration chromatography (Figure 6). When 10^{-5} M Rose Bengal was added to the reaction mixture, the mole ratio of Rose Bengal to RNA polymerase in the isolated complex was approximately 1:1. However, this stoichiometry depended upon the amount of Rose Bengal added, and additional Rose Bengal was bound to the enzyme with increasing concentrations of inhibitor. Moreover, the binding was reversible and Rose Bengal could be released from the complex by dilution, prolonged dialysis, or by repetitive passage through a Sephadex column. Thus, dilution of the Rose Bengal–enzyme complex to a concentration of less than 10^{-6} M Rose Bengal resulted in the restoration of enzyme activity.

TABLE I: Effect of Rose Bengal on Incorporation of [γ-^{32}P]ATP and [^{3}H]UMP by RNA Polymerase.[a]

Rose Bengal (M)	[γ-^{32}P]ATP Incorp (pmoles)	[^{3}H]UMP Incorp (nmoles)	[^{3}H]UMP Incorp/ [γ-^{32}P]ATP Incorp
0	4.84	4.01	828
1×10^{-6}	4.34	2.56	590
3×10^{-6}	3.50	0.73	208
1×10^{-5}	1.91	0.07	37

[a] [γ-^{32}P]ATP and [^{3}H]UMP incorporation were assayed by the procedure of Maitra *et al.* (1967) with slight modification. 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, and [^{3}H]UTP (5×10^3 cpm/nmol), 0.2 mM [γ-^{32}P]-ATP (950 cpm/pmol), 10 nmol of T7 DNA, and 10 μg of enzyme. The incubation was 20 min at 37° in the dark. The reaction mixtures were then chilled and 0.1 ml of 0.5% bovine serum albumin was added, followed by 0.1 ml of 0.1 M sodium pyrophosphate. After mixing, 3 ml of 5% trichloroacetic acid was added, mixtures were centrifuged for 5 min at 15,000*g* and pellets were dissolved in 0.2 ml of ice-cold 0.5 N NaOH. In each reaction mixture, 3 ml of 5% trichloroacetic acid were again added and the precipitate was collected by centrifugation. The washing procedure was repeated two additional times. The final acid-insoluble RNA product was isolated by filtration on glass-fiber filter, dried, and radioactivity was determined.

Discussion

The present study demonstrates that Rose Bengal inhibits DNA-dependent polymerase of *E. coli*. The inhibition does not require photooxidation since all experiments were performed in the absence of light. During photooxidation of RNA polymerase with Rose Bengal, Ishihama and Hurwitz (1969) incubated control mixtures (containing Rose Bengal and enzyme) in the dark. Such conditions resulted in no loss of enzyme activity. This can be explained by the fact that the Rose Bengal–RNA polymerase interaction is reversible. When aliquots of these control mixtures were diluted (10-fold) for assay, the concentrations of Rose Bengal in the assay mixture was less than 10^{-6} M so that no significant inhibition was detected.

The photooxidized RNA polymerase is unable to elongate RNA chains but still retains the ability of binding DNA, binding NTP, and catalyzing an extensive DNA-dependent [^{32}P]PP_i exchange reaction (Ishihama and Hurwitz, 1969). Similar properties were observed for RNA polymerase exposed to Rose Bengal in the dark. This suggests that the

TABLE II: Effect of Rose Bengal on Poly[d(A-T)]-Dependent [^{32}P]PP$_i$-Exchange Reaction.[a]

Additions	Rose Bengal (M)	[^{32}P]-PP$_i$ Incorp (nmoles)
None	0	1.5
ATP (4×10^{-6} M)	0	38.6
	1×10^{-6}	27.2
	3×10^{-6}	8.2
	1×10^{-5}	0.6
AMP (4×10^{-4} M)	0	12.5
	1×10^{-6}	12.0
	3×10^{-6}	9.1
	1×10^{-5}	0.2
ADP (4×10^{-4} M)	0	35.3
	1×10^{-6}	35.4
	3×10^{-6}	24.7
	1×10^{-5}	0.3
UpA (4×10^{-4} M)	0	25.0
	1×10^{-6}	23.4
	3×10^{-6}	17.8
	1×10^{-5}	0.2

[a] The incorpration of [^{32}P]PP$_i$ into nucleoside triphosphates was assayed by adsorption to activated charcoal (Krakow and Fronk, 1969). The complete system (0.25 ml) contained 0.08 M Tris-HCl (pH 7.8), 40 mM β-mercaptoethanol, 4 mM $MgCl_2$, 4 mM UTP, 1 mM ^{32}P-labeled sodium pyrophosphate (2×10^4 cpm/nmol), 0.1 A_{260} unit of poly[d(A-T)], and 10 μg of RNA polymerase. The incubation was 10 min at 37° in the dark. The reaction was stopped by the addition of 0.2 ml of 0.1 M EDTA (pH 6.0), and 0.1 ml of 0.1 M sodium pyrophosphate (pH 6.0), followed by addition of 0.5 ml of a 10% suspension of acid-washed, activated charcoal. After mixing, 3 ml of 0.01 M sodium pyrophosphate was added and the mixture was filtered through glass-fiber filters. The filters were washed with 40 ml of 0.01 M sodium pyrophosphate, dried, and counted.

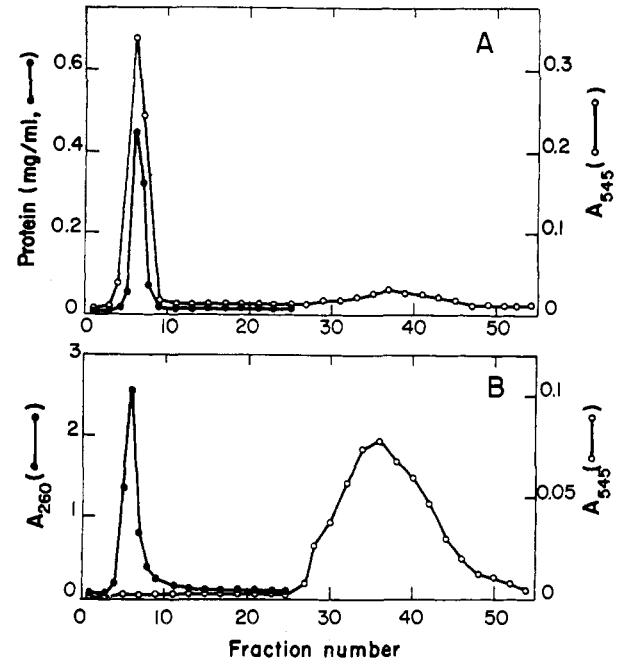

FIGURE 6: Determination of the binding of Rose Bengal to RNA polymerase (A) and to calf thymus DNA (B) by chromatography on Sephadex G-75. Reaction mixture (0.2 ml) containing 2.7 mg/ml of RNA polymerase (or 4.2 mg/ml of calf thymus DNA) and 1×10^{-5} M Rose Benagl in 0.05 M Tris-HCl (pH 8), 0.2 M KCl, 0.5 mM dithiothreitol, and 0.1 mM EDTA, was incubated at 37° for 30 min in the dark. The sample was then loaded onto a Sephadex G-75 column (1×14 cm) equilibrated and eluted with the same buffer. A control without Rose Bengal was run under the same conditions. The Rose Bengal concentration was monitored at 545 nm (○), the protein at 280 nm, and the DNA at 260 nm. RNA polymerase concentration was determined by an extinction coefficient of $A_{280}^{0.1\%} = 0.65$ (Richardson, 1966).

effects of Rose Bengal are primarily due to its binding to the enzyme rather than the sensitized photooxidation. It is conceivable that binding of Rose Bengal to RNA polymerase induces certain reversible structural changes in the enzyme which results in the loss of its ability to catalyze chain elongation. Such structural changes, however, may become irreversible after photooxidation. This is in keeping with the observation that photooxidized enzyme failed to synthesize RNA even after extensive dilution.

As has been observed by many investigators, a sigmoidal curve is obtained when the rate of RNA synthesis is plotted as a function of nucleotide concentration (Niyogi and Stevens, 1956; Anthony *et al.*, 1966, 1969). A similar effect is observed with d(A-T) copolymer as a template (Anthony *et al.*, 1969; Downey and So, 1970). However, when saturating concentrations of either ATP or UTP are used while the concentration of the alternate NTP is varied, normal Michaelis–Menten kinetics is observed. The resulting double-reciprocal plot becomes linear under these conditions and thus can be used to study inhibitor effects by kinetic analysis. The observed noncompetitive inhibition of Rose Bengal with respect to NTP (Figure 3) suggests that the inhibitor does not affect the combination of the substrates with the enzyme and a ternary inhibitor–enzyme–substrate complex may exist. Similarly, Rose Bengal did not alter the binding of RNA polymerase to DNA (Figure 4). Thus, our data suggest that Rose Bengal, NTP, and DNA occupy physically distinct sites on the enzyme. Nevertheless, it should be emphasized that the kinetic analysis described here is a simplified interpretation of a complex reaction sequence and that other independent evidence is needed to further establish this interpretation.

The most interesting result obtained in the experiments described above is the preferential inhibition of chain elongation

TABLE III: Effect of Rose Bengal on Unprimed Synthesis of Poly[(rA·rU)] and Poly(A) by RNA Polymerase.[a]

Conditions	[^{3}H]AMP Incorp (nmoles)	% Act.
Complete system[a]	7.40	100
10^{-6} M Rose Bengal	5.62	76
10^{-5} M Rose Bengal	0.12	1.6
−UTP	1.61	22
−UTP + 10^{-5} M Rose Bengal	0.18	2.5

[a] The complete system (0.25 ml) contained 80 mM Tris-HCl (pH 7.8), 4 mM β-mercaptoethanol, 2 mM $MnCl_2$, 0.6 mM UTP, 0.6 mM [^{3}H]ATP (7×10^3 cpm/nmol), and 5 μg of *E. coli* RNA polymerase. The reaction mixtures were incubated for 120 min at 37° in the dark.

(as compared to initiation) by relatively low concentrations of Rose Bengal. In addition, the [^{32}P]PP_i–NTP-exchange reactions (Table II) also indicated that Rose Bengal inhibits the formation of phosphodiester bonds (except the first one). However, it is also possible that Rose Bengal inhibits translocation of the enzyme rather than phosphodiester-bond formation. During the course of gene transcription, the polymerase must move along the template after formation of each phosphodiester bond. Failure of the enzyme to translocate will prevent the formation of subsequent bonds without affecting the first phosphodiester bond. Whether Rose Bengal inhibits enzyme translocation or phosphodiester-bond formation still remains to be resolved.

Some other inhibitors of RNA polymerase also exhibit selective effect on initiation and polymerization. Like Rose Bengal, actinomycin inhibits polymerization at low concentrations (10^{-6} M) but also inhibits initiation at much higher concentrations (10^{-5} M) (Richardson, 1969). However, unlike Rose Bengal, this antibiotic binds strongly to DNA (Kirk, 1960) and could achieve its actions by preventing RNA polymerase from moving through a region of DNA. Another antibiotic, streptolydigin attacks chain elongation by interaction with RNA polymerase (Siddhikal *et al.*, 1969; Schleif, 1969). In contrast to Rose Bengal, streptolydigin alters the affinity of UTP and CTP for polymerase (Cassani *et al.*, 1970) and stabilizes the DNA–enzyme complex (von der Helm and Krakow, 1970). Furthermore, Rose Bengal is a much more potent inhibitor. The concentration of Rose Bengal required for 50% inhibition is an order to magnitude lower than that of streptolydigin.

Evidence has been presented that Rose Bengal binds to RNA polymerase but not to DNA (Figure 7). The binding of Rose Bengal to RNA polymerase involves a strong interaction based on the K_i value obtained. Although the Rose Bengal binding markedly alters the catalytic properties of the enzyme, the binding may occur at a region distant from the substrate or template sites. Thus it will be of interest to further investigate the Rose Bengal binding sites on RNA polymerase. Such a study as approached by spectroscopic methods is presented in the following article.

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References

Anthony, D. D., Wu, C. W., and Goldthwait, D. A. (1969), *Biochemistry 8*, 246.

Anthony, D. D., Zeszotek, E., and Goldthwait, D. A. (1966), *Proc. Nat. Acad. Sci. U. S. 56*, 1026.

Burgess, R. R. (1969), *J. Biol. Chem. 244*, 6160.

Cassani, G., Burgess, R. R., and Goodman, H. M. (1970), *Cold Spring Harbor Symp. Quant. Biol. 35*, 59.

Downey, K. M., and So, A. G. (1970), *Biochemistry 9*, 2520.

Freeman, E. J., and Jones, O. W. (1967), *Biochem. Biophys. Res. Commun. 29*, 45.

Goldthwait, D. A., Anthony, D. D., and Wu, C. W. (1970), *in* First Lepetit Colloquium on RNA Polymerase and Transcription, Silvestri, L., Ed., Amsterdam, North Holland Publishing Co., p 10.

Hinkle, D. C., and Chamberlin, M. (1970), *Cold Spring Harbor Symp. Quant. Biol. 25*, 65.

Hoffee, P., Lai, C. Y., Pugh, E. L., and Horecker, B. L. (1967), *Proc. Nat. Acad. Sci. U. S. 57*, 107.

Ishihama, A., and Hurwitz, J. (1969), *J. Biol. Chem. 244*, 6680.

Kirk, J. M. (1960), *Biochim. Biophys. Acta 42*, 167.

Krakow, J. S. (1968), *Biochim. Biophys. Acta 116*, 459.

Krakow, J. S., and Fronk, E. (1969), *J. Biol. Chem. 244*, 5988.

Maitra, U., Lockwood, A. M., Dubnoff, J. S., and Guha, A. (1970), *Cold Spring Harbor Symp. Quant. Biol. 35*, 143.

Maitra, U., Nakata, Y., and Hurwitz, J. (1967), *J. Biol. Chem. 242*, 4908.

Niyogi, S. K., and Stevens, A. (1965), *J. Biol. Chem. 240*, 2593.

Richardson, J. P. (1966), *Proc. Nat. Acad. Sci. U. S. 55*, 1616.

Richardson, J. P. (1969), *Progr. Nucl. Acid Res. Mol. Biol. 9*, 75.

Schaller, H., Nüsslein, C., Bonhoeffer, E. J., Kurz, C., and Nietzschmann, I. (1972), *Eur. J. Biochem. 26*, 474.

Schleif, R. (1969), *Nature (London) 233*, 1068.

Siddhikal, C., Erbstoeszer, J. W., and Weisblum, B. (1969), *J. Bacteriol. 99*, 151.

von der Helm, K., and Krakow, J. S. (1972), *Nature (London), New Biol. 235*, 82.

Westhead, E. W. (1965), *Biochemistry 4*, 2139.

Wu, C. W., and Goldthwait, D. A. (1969), *Biochemistry 8*, 4458.

Yarbrough, L., and Hurwitz, J. (1973), in preparation.