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Rose Bengal Mediated Inhibition of DNA Polymerases: Mechanism of Inhibition of Avian Myeloblastosis Virus Reverse Transcriptase under Nonoxidative Conditions†

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ABSTRACT: DNA polymerases from eukaryotic, prokaryotic, and retroviral sources exhibit strong sensitivity (I_{50} = 5–8 μM) to Rose Bengal dye under dark (nonoxidative) conditions. The mechanism of inhibition by Rose Bengal was investigated by using avian myeloblastosis virus reverse transcriptase as a test enzyme. Rose Bengal has been found to inhibit both polymerization and reverse transcriptase associated ribonuclease H activity. The inhibition by Rose Bengal is completely reversible, the degree of inhibition being dependent on the final concentration of Rose Bengal in the reaction mixture. The kinetic analyses indicated that the inhibition is competitive with respect to substrate deoxynucleoside triphosphate and noncompetitive with respect to template-primer. The protection against Rose Bengal inhibition is afforded strictly by deoxynucleoside triphosphate that is complementary to template nucleotide. The addition of Rose Bengal to an ongoing reaction consistently inhibited DNA synthesis after a short time lag. Subsequently, studies on the kinetics of polymerization carried out by varying template to primer ratio and incubation temperature indicated that the primary action of Rose Bengal on the ongoing reaction is the prevention of reinitiation, while elongation of chains that were already initiated was unaffected. The effect of Rose Bengal on the template binding function of reverse transcriptase, using a Millipore filter binding assay procedure, provided further insight into its mechanism of inhibition, for pretreatment of enzyme with dye completely abolished the ability of the enzyme to bind to the template-primer while the stability of preformed enzyme–template complex was unaffected by Rose Bengal addition. The preliminary spectrophotofluorometric analyses of enzyme–Rose Bengal complexes indicated that the major site of Rose Bengal reactivity resides in a hydrophobic domain of the enzyme molecule, implicating this region as being responsible for stabilizing the binding of enzyme to template.

The enzymatic synthesis of DNA is a complex process and can be divided into the following steps: (a) the binding of enzyme to template-primer, (b) the binding of complementary deoxyribonucleoside triphosphate (dNTP)[1] residue to enzyme followed by the first phosphodiester bond formation between the primer terminus and bound dNTP (a process called initiation), and (c) the subsequent linking of complementary dNTPs, to form a polydeoxynucleotide (a process called elongation). In order to better understand the mechanism of catalysis of DNA synthesis and to define the structure–function relationship of DNA polymerases, we have begun an extensive characterization of various catalytic reactions excecuted by DNA polymerases with avian myeloblastosis virus (AMV) reverse transcriptase (RT) as a model DNA polymerase (Modak & Marcus, 1977a,b; Marcus et al., 1978; Modak & Srivastava, 1979; Srivastava & Modak, 1980a,b). We have developed reagents for the site-specific labeling of DNA polymerases that not only serve as active-site probes but also aid in unraveling intricacies of the enzymatic process of DNA synthesis (Srivastava et al., 1981; Modak et al., 1980). We have shown that pyridoxal 5′-phosphate is a specific inhibitor of dNTP binding and that it may be linked to a lysine residue

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[1] Abbreviations: AMV, avian myeloblastosis virus; dNTP, deoxynucleoside triphosphate; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; RB, Rose Bengal; RNase H, ribonuclease H; RT, reverse transcriptase.

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involved in the substrate binding site (Modak, 1976a; Modak & Dumaswala, 1981). This reagent was further found not to have any inhibitory effect on the AMV RT associated RNase H activity (Modak, 1976b), an observation that implied two distinct active-site structures in AMV RT. Another site-specific reagent that we have described is phenylglyoxal, which has a specificity for the template binding site (Srivastava & Modak, 1980a). The studies carried out with this and other sulfhydryl reagents and a zinc chelator, *o*-phenanthroline, have provided strong support to our hypothesis that the RNase H expression and the template binding function of RT may utilize the same site (Modak & Marcus, 1977a; Modak & Srivastava, 1979; Srivastava & Modak, 1980a,b). In our continued efforts to define various active-site structures and important structural domains in AMV RT in particular and DNA polymerases in general, we have examined the interaction of a variety of inhibitors with known specificities with several DNA polymerases (Modak et al., 1980; Srivastava & Modak, 1980b). One such reagent is Rose Bengal dye (RB), which has been used as a probe for deciphering the active-site domains of a variety of enzymes under photooxidative and nonoxidative (dark) conditions. RB has been shown to be a potent inhibitor of *Escherichia coli* RNA polymerase (Ishihama & Hurwitz, 1969; Wu & Wu, 1973a,b). The highly fluorescent nature of this dye has permitted a critical analysis of the nature of RB–RNA polymerase interaction and its effect on the various catalytic activities of that enzyme. We find it to be a strong inhibitor of a variety of DNA polymerases under both photooxidative and dark-reaction conditions. This paper describes the results of the extensive analysis of RB-mediated inhibition of AMV RT which we have used as a model DNA polymerase, under nonoxidative or dark-reaction conditions. The data clearly indicate that RB selectively inhibits reinitiation of DNA synthesis by reacting with the free enzyme species, resulting in the loss of template binding ability of AMV RT.

Materials and Methods

Materials. All the radioactive deoxyribonucleoside triphosphates were obtained from New England Nuclear, Inc. Unlabeled triphosphates and template-primers were the products of P-L Biochemicals, Inc. The molar ratio (based on total nucleotide) of template-primer was 1:1 unless indicated otherwise. Phage fd DNA was purchased from Miles Laboratories, while *E. coli* RNA polymerase holoenzyme was supplied by Enzo Biochemicals, Inc. Rose Bengal was procured from Sigma Chemical Co. Purified AMV RT was made available by the Division of Cancer Cause and Prevention, National Cancer Institute, through Dr. Joseph Beard. The DNA polymerases from viral or cellular sources were purified as described earlier (Modak & Marcus, 1977b; Modak, 1978; Srivastava & Modak, 1980a). fd DNA·[^{3}H]RNA hybrid for RNase H assays was prepared as described before (Srivastava & Modak, 1980b).

DNA Polymerase Assays. Assays were carried out in a final volume of 100 μL and contained the following components: 20 mM Hepes (pH 7.8), 1 mM dithiothreitol, 10 μg of bovine serum albumin, 20 μM appropriate [^{3}H]deoxyribonucleoside triphosphate adjusted to a final specific activity of 1000 cpm/pmol, and 0.5 μg of desired template-primer. In addition to the above mentioned standard components, different enzymes required different salt and divalent cation concentrations for the synthesis directed by various template-primers. These conditions for AMV RT are as follows: 50 mM KCl and 5 mM $MgCl_2$ with poly(rC)·$(dG)_{12-18}$ and poly(dC)·$(dG)_{12-18}$; 100 mM KCl and 10 mM $MgCl_2$ with poly(rA)·$(dT)_{12-18}$; and 100 mM KCl and 0.5 mM $MnCl_2$ with poly(dA)·$(dT)_{12-18}$. *E. coli* DNA polymerase I assays were performed with 100 mM KCl and 10 mM $MgCl_2$ with poly(dA)·$(dT)_{12-18}$ as a template-primer, while calf thymus DNA polymerase β was assayed in the presence of 100 mM KCl and 0.5 mM $MnCl_2$ with poly(rA)·$(dT)_{12-18}$ as template-primer.

Assays of terminal deoxynucleotidyltransferase were carried out with 0.5 μg of oligo$(dA)_{12-18}$ as a primer, 1 mM $MnCl_2$, and 20 M [^{3}H]dGTP as substrate (Modak, 1978, 1979).

Incubations were carried out at 37 °C for 30 min unless otherwise indicated, and the reactions were terminated by the addition of 5% (w/v) trichloroacetic acid (Cl_3CCOOH) containg 10 mM pyrophosphate. The acid-insoluble precipitate was collected on Whatman GF/B filters, washed extensively with Cl_3CCOOH, water, and finally with ethanol, dried, and counted in toluene-based scintillation fluid.

RNA Polymerase Assay. The reaction mixture contained, in a final volume of 100 μL, 80 mM Hepes (pH 7.8), 10 mM dithiothreitol, 0.5 μg of fd DNA, 1 mM each of ATP, CTP, and UTP, 20 μM [^{3}H]GTP, 50 mM KCl, 10 mM $MgCl_2$, and 2 units of *E. coli* RNA polymerase. The incubations were carried out at 37 °C for 30 min, and the acid-insoluble radioactivity was determined as above.

RNase H Assay. AMV polymerase associated RNase H assays were performed with fd DNA·[^{3}H]RNA as substrate. The reaction conditions have been previously described (Modak & Srivastava, 1979).

Nitrocellulose Filter Assay for the Binding of Template-Primer to AMV RT. The reaction mixture in a final volume of 100 μL contained the following components: 20 mM Hepes (pH 7.8), 1 mM dithiothreitol, 10 μg of albumin, 50 mM KCl, 5 mM $MgCl_2$, 150 ng of AMV RT, and 100 ng of poly(rC)·[^{3}H](dG) equivalent to 50 000 cpm. The labeling of poly(rC)·$(dG)_{12-18}$ was achieved by a short incubation (5 min) of this template-primer with high specific activity of [^{3}H]dGTP and AMV RT (reaction conditions were the same as described above) followed by salt treatment and purification of poly(rC)·[^{3}H](dG) by DEAE-cellulose chromatography. The reaction mixture was diluted with 3 volumes of filter binding buffer (20 mM Hepes (pH 7.8), 1 mM dithiothreitol, 50 mM KCl, and 5 mM $MgCl_2$) after incubation at 25 °C for 15 min. The diluted reaction mixture was immediately filtered and washed with 0.5 mL of filter binding buffer on a nitrocellulose membrane (BA-85, 13 mm, 0.45 μm; Schleicher & Schuell) at the flow rate of 15 mL/h. The membranes were dried and counted as before.

Spectrophotofluorometric Measurements. Fluorescence emission spectra of free RB and AMV RT bound RB were recorded on a Perkin-Elmer Model MPF-44B fluorescence spectrophotometer by using a quartz cuvette with a 0.5-cm light path. AMV polymerase storage buffer contained 0.2 M potassium phosphate (pH 7.2), 2 mM dithiothreitol, 50% glycerol, and 0.2% Triton X-100. The interference of Triton X-100 in fluorescence studies was overcome by two passages of enzyme solution through a 3-cm column of Bio-Bead SM-2 (20–50 mesh). The fluorescence studies were carried out with enzyme solution containing 0.2 M potassium phosphate (pH 7.2), 2 mM dithiothreitol, and 20% glycerol. Horse liver alcohol dehydrogenase (ADH) was dissolved in 0.1 M potassium phosphate (pH 7.4) and used as a standard for fluorescence studies.

All the reactions in the presence of RB were performed under dark conditions (the assay tubes were wrapped with aluminum foil to minimize the effects of light). The transfer and addition of RB were done in the dark.

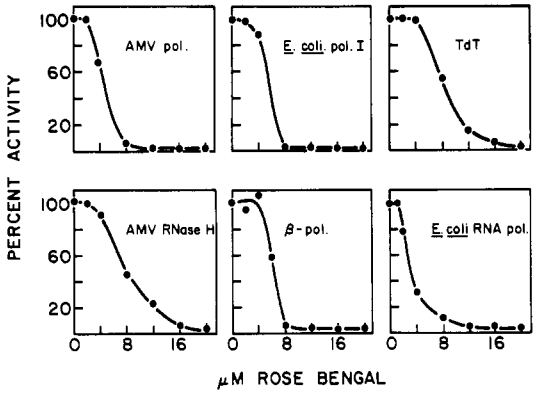

FIGURE 1: Effect of RB on the activity of various nucleic acid polymerases and AMV RT associated RNase H. The assay conditions for *E. coli* DNA polymerase I, β-polymerase, TdT (terminal deoxynucleotidyltransferase), *E. coli* RNA polymerase, and AMV RNase H were essentially as described under Materials and Methods. AMV RT was assayed with poly(rC)·$(dG)_{12-18}$ as template-primer. RB was added to individual reaction mixture at the desired concentration, and the incubations were carried out at 37 °C for 30 min in the dark (nonoxidative conditions).

Table I: Reversibility of Rose Bengal Mediated Inhibition of AMV RT[a]

final concn of Rose Bengal (μM)		activity	
during preincubation	in reaction mixture	(pmol of dGMP incorpn)	inhibition (%)
0	0	130	0
0	2	110	15
0	20	10	92
50	2	110	15
200	8	50	61
500	20	9	93

[a] A standard reaction mixture (in a total volume of 10 μL) containing 3 ng of AMV RT but no template-primer was preincubated for 5 min at 25 °C with or without RB. The entire preincubation mixture was then diluted 25-fold with standard reaction mixture that contained 0.5 μg of poly(rC)·$(dG)_{12-18}$ and 20 μM [^{3}H]dGTP. RB was then added to those tubes that did not receive it during preincubation, and enzyme activity was determined as described under Materials and Methods. The results show that inhibition of AMV RT was proportional to the final concentrations of RB in the reaction mixture.

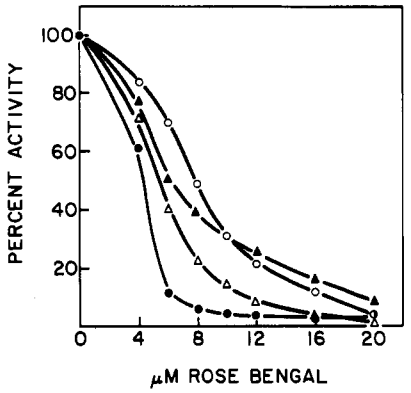

FIGURE 2: Rose Bengal mediated inhibition of AMV RT catalyzed DNA synthesis directed by various template-primers. The assay conditions for synthesis directed by various template-primers are described under Materials and Methods. The 100% activity with various template-primers was as follows: poly(rC)·$(dG)_{12-18}$ (●), 241 pmol; poly(dC)·$(dG)_{12-18}$ (▲), 20 pmol; poly(rA)·$(dT)_{12-18}$ (○), 236 pmol; and poly(dA)·$(dT)_{12-18}$ (△), 65 pmol.

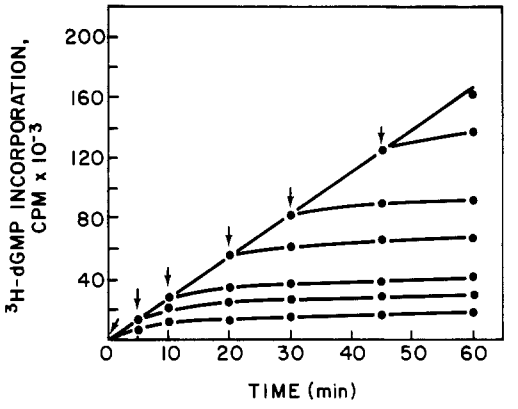

FIGURE 3: Effect of addition of RB on an ongoing DNA synthetic reaction catalyzed by AMV RT. AMV RT catalyzed synthesis of DNA was initiated with poly(rC)·$(dG)_{12-18}$ template-primer. RB was added to a final concentration of 15 μM at the desired time indicated by arrows. Aliquots of the reaction mixture were then removed at different time intervals, and acid-insoluble activity was determined as described under Materials and Methods.

Results

Sensitivity of Various DNA Polymerases to Rose Bengal. A typical dose–response curve of the effect of RB on the activity of various DNA polymerases is presented in Figure 1. A severe inhibition of *E. coli* DNA polymerase I, calf thymus DNA polymerase β, AMV RT and its associated RNase H, terminal deoxynucleotidyltransferase, and *E. coli* RNA polymerase (holoenzyme) was consistently observed in the presence of RB. The concentration of RB required for 50% inhibition (I_{50}) of the various enzymes ranged between 5 and 8 μM. The shape of the RB inhibition curves (Figure 1) and I_{50} values for various DNA polymerases are remarkably similar, suggesting a common mode of inhibition. For all the subsequent work on the characterization and analysis of the inhibitory effect of RB, we have used AMV RT as a model enzyme.

Effect of RB on the DNA Synthesis Directed by Various Template-Primers. AMV RT can utilize several synthetic and natural template-primers, and therefore, it was important to determine the extent of the inhibitory effect of RB on DNA synthesis directed by different template-primers. The results depicted in Figure 2 clearly show that synthesis directed by all the template-primers is sensitive to RB, although the dose–response patterns for sensitivity of synthesis directed by different template-primers was reproducibly different. Synthesis directed by all the template-primers was completely inhibited at RB concentrations of 20 μM and above. However, under partial inhibitory concentrations of RB, poly(rA)·$(dT)_{12-18}$ directed synthesis exhibited relatively more resistance (I_{50} = 8 μM) than that found with poly(rC)·$(dG)_{12-18}$ directed synthesis (I_{50} = 4 μM). These differences probably reflect differential affinity of template-bound enzyme to RB. No differences in this pattern were found when Mg^{2+} was substituted with Mn^{2+} (data not shown).

Properties of RB-Mediated Inhibition of AMV RT. RB inhibits AMV RT under both photooxidative (unpublished observation) and nonoxidative conditions. The important difference in the nature of the inhibition of AMV RT by RB under the two conditions is that the inhibition under the latter condition is reversible (Table I; data for photooxidation conditions not shown). It is clear that the degree of inhibition is dependent upon the final concentration of RB in the reaction mixture, for merely by dilution of inhibited reactions, rates equivalent to those of control reactions may be restored (data not shown). The effect of RB on the rates of dNTP polymerization in an ongoing reaction is shown in Figure 3. Ad-

Table II: Effect of Substrate Triphosphate and Template-Primers on the Rose Bengal Mediated Inhibition of AMV RT[a]

template-primer	dNTP	pmol of dNTP incorpn: no RB	pmol of dNTP incorpn: 15 μM RB	inhibition (%)
poly(rC)·(dG)$_{12-18}$				
0.5 μg	20 μM dGTP	190	2	99
5 μg	20 μM dGTP	210	4	98
0.5 μg	2 mM dGTP	1020	970	5
0.5 μg	20 μM dGTP, 2 mM TTP	120	10	92
poly(rA)·(dT)$_{12-18}$				
0.5 μg	20 μM TTP	110	30	73
5 μg	20 μM TTP	140	35	75
0.5 μg	2 mM TTP	410	370	10
0.5 μg	20 μM TTP, 2 mM dGTP	90	30	67

[a] Standard reaction mixtures contained 3 ng of AMV RT, indicated amounts of template-primer and substrate or nonsubstrate dNTP, and enzyme activity was determined in the presence and absence of 15 μM RB. Assay conditions were as described under Materials and Methods.

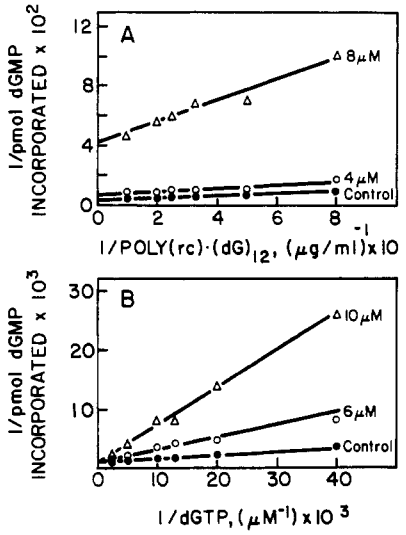

FIGURE 4: Double-reciprocal plot of 1/velocity vs. 1/[poly(rC)·(dG)] (A) and 1/[dGTP] (B) with two different concentrations of RB. The units of velocity are expressed as picomoles of dGMP incorporated under standard assay conditions (see Materials and Methods).

dition of RB at any time during catalysis results in the inhibition of catalysis following a slight but reproducible time lag. Up to a 10-fold change in either enzyme or template-primer concentration did not alter the degree of RB inhibition, while increasing substrate (but not nonsubstrate) dNTP concentration had a significant protective effect against RB inhibition of AMV RT (Table II).

Mechanism of Inhibition. A kinetic analysis of the effects of RB on DNA synthesis was carried out under standard conditions with varying poly(rC)·(dG)$_{12-18}$ or dGTP concentrations in the presence of two different RB concentrations. The results are presented in the form of double-reciprocal plots of velocity vs. template-primer (Figure 4A) or substrate triphosphate concentrations (Figure 4B) which show that the RB inhibition is competitive with respect to substrate dNTP and noncompetitive with respect to template-primer. The K_i for RB was calculated to be 1.5 μM. A modified Scatchard plot from the dose–response and inhibition data (Rugg et al., 1978) was also constructed to determine the probable number of reactive sites on the enzyme. The results indicated that RB

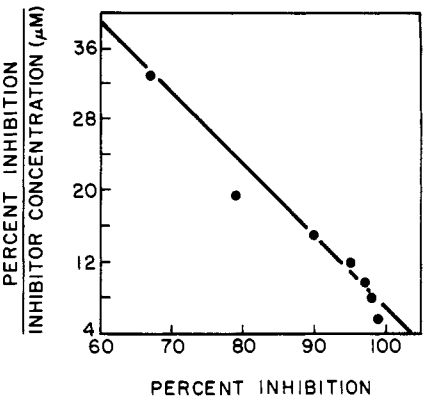

FIGURE 5: Modified Scatchard plot for the inhibition of AMV RT by RB. RB was added to individual reaction mixtures at desired concentrations. The AMV RT assay was carried out with poly-(rC)·(dG)$_{12-18}$ template-primer. The incubations were carried out at 37 °C for 30 min, and insoluble radioactivity was determined as described under Materials and Methods.

Table III: Effect of Substrate dNTP and Substrate dNTP Site-Directed Reagents on the Inhibition of AMV RT Associated RNase H Activity by Rose Bengal[a]

additions	activity (pmol of nucleotide solubilized/ 60 min)	inhibition (%)
none (control)	28	0
25 μM RB	<1	99
25 μM RB + 2 mM dNTPs[b]	4	86
25 μM RB + 1 mM pyridoxal 5'-phosphate	3	89
25 μM RB + 1 mM sodium pyrophosphate	2	93

[a] AMV RT associated RNase H activity was assayed in a standard reaction mixture containing substrate dNTPs or substrate dNTP site-directed reagents (pyridoxal 5'-phosphate or pyrophosphate) in the presence and absence of 25 μM RB. fd DNA·[^{3}H] RNA was used as substrate for RNase H assays as described under Materials and Methods. [b] 2 mM each of dATP, dCTP, dGTP, and dTTP; the concentration of $MgCl_2$ was 15 mM.

reacts with a single class of reactive site on the enzyme (Figure 5). A K_i of 1.0 μM was also confirmed from this plot. A low K_i for the inhibitor compared to a high K_m (60 μM) for the substrate dNTP implies a strong affinity of RB at or near the substrate binding site and also provides some explanation for the high substrate concentration required for the observation of a protective effect (Table II).

Effect of RB on the Template Binding Function. The studies described above implicated the substrate binding site as a possible target for the reaction of RB with enzyme. However, action of RB on AMV RT differs from several other substrate binding site directed reagents, e.g., pyridoxal 5'-phosphate and pyrophosphate (Modak, 1976a,b; Srivastava & Modak, 1979), in that RB also inhibits expression of AMV RT associated RNase H activity (Figure 1). Neither by pretreatment of enzyme with substrate binding site directed reagents nor by inclusion of high concentration of all four substrate dNTPs could RNase H activity be protected from RB inhibition (Table III). We, therefore, conclude that RB-mediated inhibition of AMV RT (a) is affected via a mechanism that is distinct from classical substrate binding site directed reagents and (b) possibly occurs through a site that controls both substrate binding and template binding. Since substrate binding and subsequent polymerization by AMV RT are dependent on the prior binding of template-primer, failure

Table IV: Effect of Rose Bengal on the Template Binding Capacity of AMV Reverse Transcriptase

incubation conditions	retention of radioactivity[a] (cpm × 10^{-3})	template binding (%)
enzyme + RB[b] (μM)		
0	19	100
0.5	18	95
5	8	42
10	<1	3
enzyme-template + RB[c] (μM)		
0	22	100
0.5	20	91
5	18	82
10	17	77

[a] On nitrocellulose filter. [b] The reaction mixture containing 150 ng of AMV RT was preincubated for 15 min at 25 °C with the desired concentration of RB before addition of poly(rC)·[^{3}H](dG) (50 000 cpm). [c] The preincubation of AMV RT with poly(rC)·[^{3}H](dG) was carried out for 15 min at 25 °C before the addition of the desired concentration of RB. The reaction mixture and filter binding assay conditions were as described under Materials and Methods.

or sensitivity in the latter function could conceivably result in the failure to bind appropriate substrate. We therefore investigated the effect of RB on the template binding function of AMV RT. A Millipore filter binding assay was standardized for this analysis where the binding of radioactive template-primer [e.g., poly(rC)·[^{3}H](dG)] to filter depended on the complex formation between enzyme and template-primer. It is clear from the results (Table IV) that enzyme pretreated with RB lost its ability to bind to the template-primer whereas the addition of RB to preformed template-primer–enzyme complexes had very little or no dissociating effect on such complexes. Thus, the inhibition of RNase H activity as well as polymerase activity could be explained on the basis of the inability of RB treated enzyme to bind to polynucleotide templates. The reversible nature of enzyme inactivation by RB was also confirmed by using the template binding assay. A dilution of RB pretreated enzyme in the reaction mixture lacking RB resulted in the prompt binding of template-primer (data not shown). This unique reactivity of RB toward enzyme also seemed to explain the lag that we observed in the inhibitory effect on DNA synthesis when RB was added to an ongoing reaction (Figure 3). Apparently, an enzyme engaged in the catalysis of DNA synthesis would not exhibit reactivity toward RB until the completion of the previously initiated chain. However, upon completion of the chain, it would transiently dissociate from the template-primer and would react with RB resulting in the failure of such species to reinitiate DNA synthesis. The results of the time course of DNA synthesis and the effects of RB addition on these reactions are consistent with this notion (Figure 3). We therefore carried out rather detailed analysis of this inhibition to clarify the differential effects of RB addition on the initiation and elongation process in the enzymatic synthesis of DNA catalyzed by AMV RT.

Effect of RB on the Initiation/Elongation of DNA Synthesis Catalyzed by AMV RT. We compared the time course of DNA synthesis with two different poly(rC)·$(dG)_{12-18}$ template-primers with a template-primer molar ratio (based on total nucleotide) of 1:1 and 20:1. It was expected that the frequency of reinitiation of synthesis will depend upon the number of priming sites available to a nonsaturating amount of enzyme. Thus, a template-primer with a 1:1 molar ratio

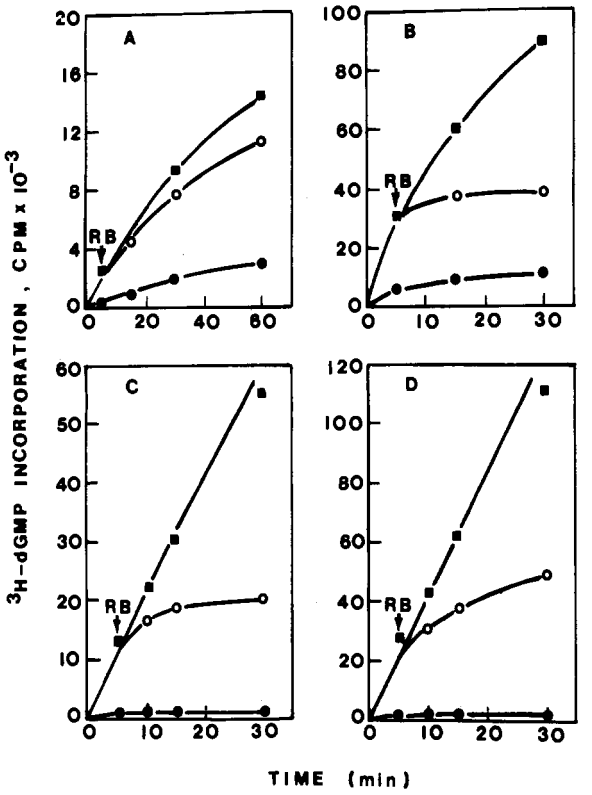

FIGURE 6: Effect of addition of RB on the rates of AMV RT catalyzed poly(dG) synthesis at different temperatures and with varying ratios of template-primer. Frames A and B show the effect of RB addition prior to (●) and 5 min after the initiation of DNA synthesis (○) on poly(rC)·$(dG)_{12-18}$ template-primer by AMV RT at the incubation temperature of 10 (A) or 37 (B). Frames C and D depict the rate of DNA synthesis directed by two different poly(rC)·$(dG)_{12-18}$ template-primers with molar nucleotide ratios of template-primer being 1:1 (C) and 20:1 (D). RB was present at a final concentration of 15 μM. The control samples (■) did not contain RB. The incubations, except for set A, were carried out at 37 °C in the dark. Aliquots were withdrawn at desired times, and acid-insoluble counts were determined as described under Materials and Methods. The arrows indicate the time of addition of RB.

will have small gaps to fill and will require more frequent reinitiation than the template-primer that has longer gaps due to the relatively limited amount of primer (e.g., 20:1 ratio). Thus, when the effect of the addition of RB to poly(rC)·$(dG)_{12-18}$ with a template-primer molar ratio of 1:1 and 20:1 was examined (Figure 6C,D), inhibition of catalysis was much more rapid with equimolar template-primer than that observed with 20:1 ratio species. Another factor that will influence the rate of initiation events is the temperature of incubation. At suboptimal temperatures, elongation rates are severely decreased, and therefore, enzyme may be expected to remain associated with template-primer (growing chain) for a longer time, while, at optimal temperature, the rate of dNTP turnover is rapid, and therefore, enzyme may reinitiate synthesis more frequently. Thus, dissociation of template-primer and enzyme would occur less frequently at lower temperature, and threfore, addition of RB should have much less of an inhibitory effect when addition is carried out at optimal temperature. A time course of synthesis directed by poly(rC)·$(dG)_{12-18}$ in the presence and absence of RB at two temperatures clearly shows that the addition of RB to an ongoing reaction at 10 °C has very little inhibitory effect in contrast to almost complete inhibition of DNA synthesis at 37 °C (Figure 6A,B).

Spectrophotofluorometric Analysis of RB–AMV RT Complex. The highly fluorescent nature of RB has been used to good advantage to probe the nature of reactive sites on the

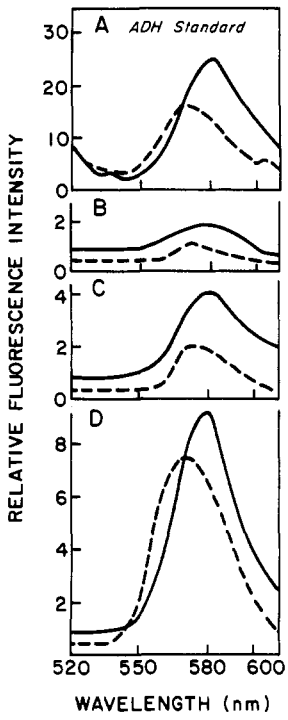

FIGURE 7: Fluorescence emission spectra of free (broken lines) and enzyme-bound (solid lines) RB. Emission spectra of free RB and ADH-bound RB (frame A) were recorded at the excitation wavelength of 500 nm. The concentrations of ADH and RB were 1.6×10^{-6} and 1×10^{-6} M, respectively. Emission spectra for AMV RT bound RB were recorded at 0.1×10^{-6} (frame B), 0.25×10^{-6} (frame C), and 0.5×10^{-6} M (frame D) concentrations of RB when the excitation wavelength was set at 500 nm. The concentration of AMV RT was constant at 0.33×10^{-6} M.

enzymes sensitive to this dye. In case of *E. coli* DNA dependent RNA polymerase, which is sensitive to RB, Wu & Wu (1973a) have carried out an elegant analysis of interaction of this enzyme with RB by spectrofluorometric measurements. Similar types of analysis have also been carried out with another sensitive enzyme, alcohol dehydrogenase (Brand et al., 1967). Although the limited quantities of AMV RT that were available to us restricted our analyses to a rather low range of sensitivity, we attempted such preliminary analyses to obtain qualitative information on the nature of RB–AMV RT interaction. Alcohol dehydrogenase was used as a control enzyme to establish proper spectrofluorometric analyses in these studies, and results obtained with AMV RT were compared to the patterns reported in the literature with RNA polymerase as a test enzyme (Wu & Wu, 1973a). The emission spectra of free RB and RB–AMV RT are shown in Figure 7. Upon excitation at 500 nm the emission maximum of free RB was seen at 570 nm. When RB was bound to enzyme, the emission maximum shifted to 580 nm with the increase in the fluorescence emission of RB. We have consistently (three independent experiments) observed this effect at three different concentrations (0.1, 0.25, and 0.5 μM) of RB, and this effect is similar to that observed with alcohol dehydrogenase (Figure 6) and that published for RNA polymerase (Wu & Wu, 1973a). The quantitation of RB bound to enzyme protein could not be obtained with the currently available amounts of enzyme protein. Spectrophotofluorometric analysis further indicates that the region of AMV RT binding RB is probably hydrophobic in nature.

Discussion

The present study demonstrates that RB is a potent inhibitor of DNA polymerases (Figure 1) and that its inhibitory action is most likely due to interference in the template binding function of these enzymes. Rose Bengal has been found to irreversibly inactivate DNA polymerases in the presence of light (unpublished observations). However, under dark conditions, the inhibition by RB is completely reversible as judged by catalytic activity as well as by template binding assays (Table I). The kinetic analysis of such inhibition indicated that the substrate binding sites may be a possible site for RB action. However, the substrate binding site per se has been ruled out as a target for RB reactivity for the following reasons: (a) Substrate binding site directed reagents, viz., pyridoxal 5′-phosphate (Modak, 1976a,b; Modak & Dumaswala, 1981) and pyrophosphate (Srivastava & Modak, 1979), have no effect on RNase H activity in contrast to RB which inhibits both substrate polymerization and nucleolytic reactions (Figure 1). (b) Pretreatment of AMV RT with these reagents or inclusion of substrate dNTPs do not protect against RNase H inhibition by RB. Therefore, the effect of RB on the substrate binding and polymerization was concluded to be a consequence of blockage of some other process that we have subsequently identified as a template binding function which is a known prerequisite for the binding of substrate dNTP (Kornberg, 1980). Indeed, the inhibition of RNase H activity by RB may be explained on the basis inability of RB-treated enzyme to bind its polynucleotide substrate. The apparent protection afforded by rather high concentrations of complementary dNTP may be explained by the increased stability of the template–enzyme complex under this condition. High concentrations of dNTPs have been shown to generate longer complementary DNA products on RNA templates in vitro due to the prevention of premature termination (Haseltine et al., 1976; Rothenberg & Baltimore, 1977).

A kinetic study of the changes in rates of DNA synthesis upon RB addition to an ongoing reaction provided an additional indication that inhibition of catalysis by AMV RT required dissociation of enzyme from template-primer. When the effect of RB on the template binding capability of AMV RT was directly examined by filter binding assays, it was noted that pretreatment of enzyme with RB resulted in the failure of enzyme to form a complex with template-primer, while performed enzyme–template complex rendered considerable resistance to RB addition. These observations provide the explanation for the short but consistent time lag that we have observed in the inhibition of ongoing DNA synthesis upon RB addition (Figure 3). Thus, it appears that a preformed enzyme–template-primer complex supports chain elongation in the presence of RB until it completes the initiated chain. It is during the transition period when enzyme dissociates from the completed chain and reinitiates at other sites that it would react with RB and become unable to bind at a new initiator site. Ample experimental evidence to support this explanation has been provided by monitoring the time course of DNA synthesis catalyzed by AMV RT using templates containing multiple and limited primers and by varying incubation temperatures (Figure 6A–D). These studies confirm that RB does not inhibit elongation of chains that are duly initiated but inhibits the process of reinitiation. One possible use of these observations may be in the determination of processivity of a desired DNA polymerase on a given template-primer. The determination of the product size in an ongoing reaction and the effect of RB addition on the size of the product should reveal the number of nucleotides that enzyme may polymerize upon a single initiation.

The effect of RB on *E. coli* RNA polymerase differs from that which we have shown for AMV RT. In the case of *E.*

coli RNA polymerase, the major effect of RB was found to be on the process of elongation while initiation of RNA synthesis remained unaffected (Wu & Wu, 1973b). Moreover, the addition of RB to an ongoing reaction caused instantaneous inhibition of RNA synthesis. It was only at very high concentrations of inhibitor that the binding of enzyme to phage T7 DNA template and initiation of RNA synthesis were affected. The apparent differences in the response of DNA and RNA polymerases to RB may be due to the subtle differences in the nature of initiation of nucleic acid synthesis by these two nucleotide polymerizing enzymes. While initiation of DNA synthesis occurrs via binding of an enzyme to a *preexisting* primer terminus, RNA polymerases bind to DNA and initiate RNA synthesis by aligning a complementary purine nucleotide which then serves as the primer for subsequent chain elongation. It is only after this step has occurred that the RNA polymerase reaction could be considered equivalent to "initiation" by DNA polymerases.

Since the RB-mediated inhibition of DNA synthesis catalyzed by AMV RT appears due to impairment of the template binding function of the enzyme, it was of interest to explore the nature of the RB reactive site on the enzyme molecule. With RB being a strongly fluorescent dye, extensive analysis of its interaction with a desired enzyme protein has been reported in the literature (Brand et al., 1967; Wu & Wu, 1973a). Preliminary data on the fluorescence emission spectrum of free RB and RB–enzyme complex clearly indicated an increase in the emission of RB with a red shift when RB was bound to enzyme (Figure 7). This characteristic of RB–AMV RT is quite similar to that reported for *E. coli* RNA polymerase (Wu & Wu, 1973a) and implies basic similarity in the interaction of these two enzymes with RB. Further quantitative analysis could not be carried out due to the unavailibility of milligram quantities of homogeneous AMV RT. We, threfore, conclude that similar to RNA polymerase, the environment of the binding site of RB on AMV RT is most likely of a nonpolar nature and probably consists of a hydrophobic pocket.

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