Interaction of Purified Ribonucleic Acid Directed Deoxyribonucleic Acid Polymerase of Avian Myeloblastosis Virus and Murine Sarcoma–Leukemia Virus with a Rifamycin SV Derivative†

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ABSTRACT: The rifamycin SV derivative, 2,5-dimethyl-4-*N*-benzyldemethylrifampicin (AF/ABDMP), inhibited the purified RNA directed DNA polymerase of avian myeloblastosis virus (AMV) and the murine sarcoma–leukemia virus, Moloney strain [M-MSV(MLV)], by binding reversibly to the enzyme and not to RNA or DNA templates. The inhibitory effect of the drug could be overcome by the addition of excess enzyme but not of excess template, and the inhibition could be completely reversed by dilution of the drug–enzyme mixture. From Lineweaver–Burk analysis, the inhibition by AF/ABDMP is noncompetitive with respect to template and deoxyribonucleoside triphosphate substrates. Thus, the drug binds to a site different from the catalytic site for template and substrates. Preincubation of the AMV DNA polymerase with template before the addition of drug reduced the inhibition by AF/ABDMP. When high concentrations of the drug were added during polymerization, DNA synthesis continued unabated for at least 1 min, before inhibition was observed. These results suggest that the drug acts at an early step(s) in DNA synthesis. The effect of different concentrations of AF/ABDMP on the initial rate of DNA polymerization suggests a cooperative binding of more than one drug molecule per enzyme molecule, since the ratio of the velocity measured in the absence of drug to the velocity measured in the presence of drug was not linearly dependent on the drug concentration. These results were obtained with both the AMV DNA polymerase with two subunits, α and β which have molecular weights of 65,000 and 105,000 daltons, respectively, and with AMV DNA polymerase which has a single polypeptide chain, α, with a molecular weight of 65,000 daltons. A Hill plot of the kinetic data gave two slopes with n values of 3 and 7 with $\alpha\beta$ AMV polymerase, the change in slope occurring at about 60% inhibition. A straight line was observed with α AMV DNA polymerase with an n value of 6.5. A Hill coefficient of 1 was obtained when *Escherichia coli* RNA polymerase was analyzed in the same manner. The possibility that the drug binds to a hydrophobic site on the enzyme which exerts a regulatory function on the initiation of DNA synthesis is discussed.

Suitable modifications of the side chain of the antibiotic rifampicin transforms this compound into an inhibitor of the DNA polymerase activities of several RNA tumor viruses (Gurgo *et al.*, 1971; Green *et al.*, 1971). Subsequent studies have revealed numerous rifamycin derivatives which inhibit viral RNA directed DNA polymerase and DNA directed RNA and DNA polymerases of mammalian cell, animal, virus, and bacteriophage origin (Szilagyi and Pennington, 1971; Gurgo *et al.*, 1972; Green *et al.*, 1972a,b; Meilhac *et al.*, 1972; Yang *et al.*, 1972; Chamberlin and Ring, 1972).

At least three of these derivatives, including AF/ABDMP,[1] appear to be specific for polymerizing enzymes since they have been found inactive against a variety of nonpolymerizing enzymes (Gerard *et al.*, 1973). Several rifamycin derivatives were found to block cell transformation by murine sarcoma virus (Calvin *et al.*, 1971; Green *et al.*, 1972a; Ting *et al.*, 1972) and to delay the appearance of tumors induced by chemical carcinogens (Joss *et al.*, 1973).

AF/ABDMP, the first derivative found to inhibit the viral reverse transcriptase, has been the most extensively studied. The drug inhibits focus formation of Balb/3T3 cells infected with M-MSV(MLV) (Calvin *et al.*, 1971). It has been observed that the complete structure of this compound, and not the side chain component by itself, is required for inhibition of the viral enzyme (Gurgo *et al.*, 1971). In this communication, we report studies on the mechanism of inhibition by AF/ABDMP of purified DNA polymerase of two RNA tumor viruses which show that the drug interacts with the viral polymerase at a site different from the active site of the enzyme in a manner that differs from that found with RNA polymerase molecules of bacterial origin.

Materials and Methods

Materials. The rifamycin SV derivative, AF/ABDMP, kindly provided by Dr. Sensi and G. Lancini of Gruppo Lepetit, Milan, Italy, was dissolved in Me_2SO at 10 mg/ml and stored at 4°. NP-40 was obtained from Shell Chemical Co.; [^{3}H]TTP from New England Nuclear Co.; unlabeled deoxyribonucleoside triphosphates from Sigma Chemical Co.; DEAE-cellulose disks from Reeve Angel; poly(A) and oligo(dT) (12–18 nucleotides long) from Collaborative Research; and poly(dA-dT) from Miles Laboratories.

Viruses. AMV was generously provided by Dr. Joseph Beard, Duke University, and was purified as described pre-

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[1] Abbreviations used are: M-MSV(MLV), murine sarcoma–leukemia virus; AF/ABDMP, 2,5-dimethyl-5-*N*-benzyldemethylrifampicin; NP-40, Nonidet P-40; AMV, avian myeloblastosis virus.

viously (Green *et al.*, 1970). The M-MSV(MLV) was grown and purified in a similar manner.

Purification of Polymerases. The α and $\alpha\beta$ RNA directed DNA polymerases of AMV were purified according to Grandgenett *et al.* (1973) through the phosphocellulose step of the procedure. Unless otherwise specified, the AMV polymerase used throughout this study was the $\alpha\beta$ polymerase. For some experiments, $\alpha\beta$ AMV polymerase carried through the additional step of glycerol gradient centrifugation was used. The enzyme was approximately 90% pure after this step. The α DNA polymerase contained a single subunit with a molecular weight of 65,000 daltons and $\alpha\beta$ DNA polymerase had two subunits of 65,000 and 105,000 daltons, as determined by sodium dodecyl sulfate polyacrylamide disc gel electrophoresis. Purification of the M-MSV(MLV) RNA directed DNA polymerase was carried out by zonal centrifugation of 1% NP-40 lysed virus in 15–30% sucrose gradients (12.5 ml) containing 10 mM sodium phosphate buffer (pH 7.5), 2 mM $MgCl_2$, 10 mM dithiothreitol with 5% glycerol. The virus lysate was centrifuged in an SW 41 rotor at 40,000 rpm for 24 hr at 4°. Fractions were collected from the bottom of the centrifuge tube and assayed for DNA polymerase activity using poly(dA-dT) as template. Over 80% of the polymerase activity was found as a major peak sedimenting at 4.3 S. The peak DNA polymerase fractions were pooled and stored in 30% glycerol at −20°. The purified enzyme was free of endogenous DNA polymerase activity.

The *Escherichia coli* DNA directed RNA polymerase with σ factor was purified through one glycerol gradient centrifugation (Burgess, 1969).

Polymerase Assays. The DNA polymerase activity of AMV polymerase was assayed at 37° in the following reaction mixture (0.1 ml): 45 mM Tris-HCl (pH 8.3), 3 mM dithiothreitol, 4 mM $MgCl_2$, 30 mM NaCl, 0.08 mM each of dATP, dCTP, and dGTP, and 0.01–0.1 mM [^{3}H]TTP (15–20 μCi per assay). Poly(dA-dT) at 20 μg/ml or poly(A)-oligo(dT) (20 and 1 μg/ml, respectively) were used as templates unless otherwise indicated. The reaction was terminated by the addition of EDTA to a final concentration of 20 mM. The amount of radioactive polymer formed was determined with Whatman DEAE-cellulose (DE-81) paper disks (Blatti *et al.*, 1969). The enzyme was diluted prior to enzyme assays in 0.2 M potassium phosphate buffer (pH 8.0), 2 mM dithiothreitol, 0.1 mM EDTA, and 10% glycerol (AMV diluting buffer), and 10-μl aliquots were added per assay.

The reaction mixture (0.1 ml) for the M-MSV(MLV) DNA polymerase contained 40 mM Tris-HCl (pH 8.0), 3 mM dithiothreitol, 30 mM NaCl, 2.5 mM $MgCl_2$, 0.1 mM each of dATP, dGTP, and dCTP, and 0.01 mM [^{3}H]TTP (10–20 μCi/assay). Poly(dA-dT) at 20 μg/ml was used throughout unless otherwise indicated. The diluting buffer for the MSV polymerase contained 10 mM sodium phosphate (pH 7.5), 2 mM $MgCl_2$, 10 mM dithiothreitol, and 30% glycerol; 30 μl of buffer containing variable amounts of viral DNA polymerase were added per assay.

The assay for *E. coli* RNA polymerase was performed as described by Lill *et al.* (1970) with some modifications. The preincubation mixture (0.2 ml) contained 12.5 μg of *E. coli* RNA polymerase, 50 mM Tris-HCl (pH 8.0), 8 mM $MgCl_2$, 1 mM dithiothreitol and different amounts of AF/ABDMP. The reaction was initiated by adding 0.2 ml of substrate mixture prewarmed at 37° containing Tris-HCl and $MgCl_2$ at the above concentrations plus 6×10^{-4} M ATP, 6×10^{-5} M [^{3}H]UTP (20 μCi), and poly(dA-dT) (25 μg/ml).

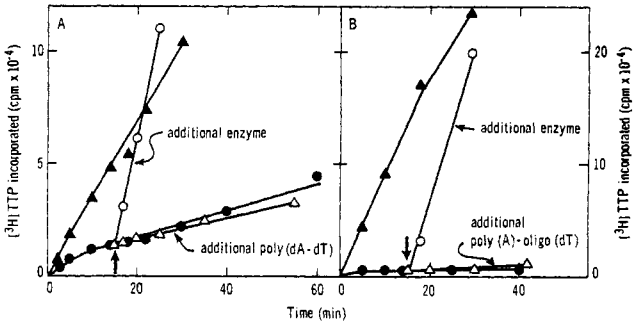

FIGURE 1: The effect of additional DNA polymerase or template on AMV polymerase activity. (A) A 2-ml reaction mixture containing poly(dA-dT) (2 μg/ml) and AF/ABDMP (1.08×10^{-5} M) was started by the addition of AMV polymerase (3 μg/ml). After 12 min of incubation the reaction mixture was divided into three equal fractions. At 15 min additional enzyme (○) or template (△) were added to separate fractions at 7.5 and 100 μg/ml, respectively, with one fraction remaining as drug control (●). At the indicated times, 90-μl samples were removed and assayed as described in Materials and Methods. The control curve (▲) was obtained with Me_2SO present instead of AF/ABDMP. (B) An identical experiment was performed as above except that the reaction mixture contained poly(A)-oligo(dT) (20:1 μg/ml) and AF/ABDMP (2.6×10^{-5} M). The reaction was initiated by the addition of AMV polymerase (0.6 μg/ml). Additional enzyme (2.5 μg/ml) (○) and poly(A)-oligo(dT) (△) (140 and 7 μg/ml, respectively) were added as previously described. The drug control (●) and Me_2SO (▲) have been described.

Results

Effect of Enzyme or Template Addition During Polymerization on the Reversible Inhibition by AF/ABDMP. AF/ABDMP, at a concentration of $2–4 \times 10^{-5}$ M, inhibited completely the polymerase activity of AMV DNA polymerase. The inhibition was completely reversible since suitable dilution of the drug–enzyme mixture completely restored enzymatic activity (Table I). Similar results were obtained with M-MSV-(MLV) DNA polymerase (data not shown).

TABLE I: Reversal of AF/ABDMP Inhibition by Dilution.[a]

	[^{3}H]TTP Incorporated Before Dilution	[^{3}H]TTP Incorporated After Dilution	% Recovery of Activity After Dilution
Control 2% Me_2SO	60	74	123
AF/ABDMP, 2×10^{-5} M	5.9	58	98
AF/ABDMP, 2.6×10^{-5} M	1.24	59	99
AF/ABDMP, 3.2×10^{-5} M	0.77	66	110

[a] AMV polymerase at a concentration of 3 μg/ml was added to four assay mixtures containing poly(A)-oligo(dT) and Me_2SO or AF/ABDMP at the above molar concentrations. After 1 min incubation at 37°, 50 μl was taken from each tube and diluted ten times with a complete assay mixture containing bovine serum albumin (200 μg/ml) but lacking Me_2SO or drug. Aliquots were taken from these diluted samples and the undiluted controls after 0, 5, and 10 min of incubation and assayed as previously described. Incorporation is expressed as cpm $\times 10^4$/10 min incubation.

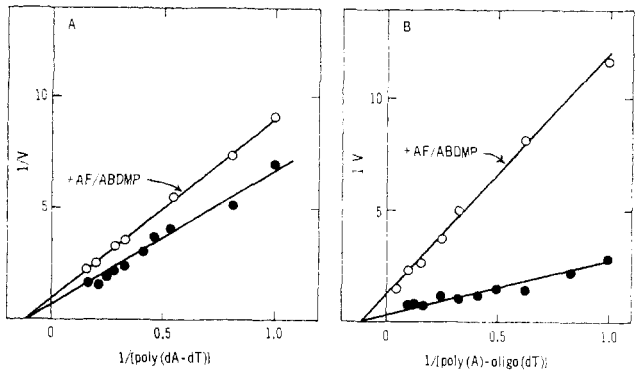

FIGURE 2: Lineweaver–Burk plot of AMV DNA polymerase activity as a function of template concentration in the presence or absence of AF/ABDMP. (A) A standard reaction mixture containing AF/ABDMP (○) (1.08×10^{-5} M) was distributed in a series of tubes containing poly(dA-dT) at a final concentration which ranged from 0.4 to 12 μg/ml. A parallel experiment using Me_2SO (●) instead of AF/ABDMP was used as a control. The reaction was started by the addition of AMV polymerase (7.5 μg/ml). Aliquots were taken after 3, 6, and 9 min, and initial velocities were determined. (B) An additional experiment was performed as described above except AF/ABDMP (2.6×10^{-5} M) (○) and poly(A)-oligo(dT) were used. Poly(A) ranged in concentration from 1 to 80 μg/ml with the amount oligo(dT) maintained at a ratio of 1:20 with respect to the amount of poly(A). The reaction was started by the addition of enzyme (0.6 μg/ml). Me_2SO (●) was present in the control reaction mixture.

TABLE II: Inability of Added Template to Reverse Inhibition of Polymerase Activity by AF/ABDMP.[a]

Poly(dA-dT), μg/ml	% Inhibition	
	AMV Polymerase	M-MSV(MLV) Polymerase
10	68	ND
20	60	50
30	70	54
40	ND[b]	60
60	ND	60
100	70	60
160	65	ND

[a] Increasing concentrations of poly(dA-dT) were distributed in a series of tubes containing assay mixtures for AMV DNA polymerase and AF/ABDMP (1.08×10^{-5} M). The reaction was initiated by the addition of AMV enzyme (8.0 μg/ml). M-MSV(MLV) polymerase was preincubated in diluting buffer (see Materials and Methods) with AF/ABDMP for 5 min at 37°. The reaction was initiated by the addition of substrate mixture and increasing concentrations of poly(dA-dT). The final concentration of the enzyme was 2 μg/ml, that of the drug was 2.3×10^{-5} M. The reactions were carried out for 30 min at 37°. The per cent inhibition was calculated on the basis of control incubation which included Me_2SO instead of AF/ABDMP. [b] ND = not determined.

Under conditions of partial inhibition with limited amounts of drug, it is possible to examine whether the addition of excess enzyme or template can overcome drug inhibition. As shown in Figure 1A and 1B, the addition of enzyme (a 2.5-fold increase) after a 15-min incubation caused a rapid increase in the rate of DNA polymerization. In contrast, a 50-fold increase in poly(dA-dT), and an eight fold increase in poly(A)-oligo(dT) did not affect the reaction. These data suggest that AF/ABDMP binds to the enzyme molecule and not to the template.

Effect of AF/ABDMP on the Initial Rate of DNA Synthesis as a Function of Template or Deoxyribonucleoside Triphosphate Concentration. To substantiate that AF/ABDMP does not interact with the template, the effect of increasing concentrations of poly(dA-dT) or poly(A)-oligo(dT) on polymerase inhibition by a constant amount of AF/ABDMP was studied (Figures 2A and 2B). Lineweaver–Burk plots showed no competition between drug and template. Even when poly(dA-dT) concentrations above saturating levels were used, the degree of inhibition of both AMV and M-MSV-MLV polymerase remained constant (Table II). Therefore, inhibition cannot be a consequence of binding of drug to the template. A similar argument has been used to demonstrate the binding of streptolidigin to the *E. coli* RNA polymerase (Cassani *et al.*, 1971).

We previously reported that AF/ABDMP is a noncompetitive inhibitor for the binding of each of the four deoxyribonucleoside triphosphates to the endogenous primed M-MSV-(MLV) polymerase (Green *et al.*, 1972b). In further experiments we analyzed the effect of AF/ABDMP on the kinetics of incorporation of TTP and dATP into DNA by purified AMV and M-MSV(MLV) polymerase. Initial rates were determined by multiple point assays at various deoxyribonucleoside triphosphate concentrations in the absence and presence of drug, and the data were analyzed on Lineweaver–Burk plots similar to those in Figure 2A and 2B. The drug was found to be a noncompetitive inhibitor for the incorporation of dATP and TTP with M-MSV(MLV) polymerase with poly(dA-dT) as template and for the incorporation of TTP with AMV polymerase using poly(A)-oligo-(dT). In each case the V_{max} was reduced in the presence of the drug but the K_m remained unchanged. The following apparent K_m values were obtained: 3.3×10^{-5} M for TTP with the AMV polymerase and poly(A)-oligo(dT) as template, and 1.0×10^{-5} M for TTP and 0.5×10^{-5} M for dATP with M-MSV(MLV) polymerase using poly(dA-dT) as template.

Template Protection of AMV DNA Polymerase Activity. From the data observed in Figure 2A and 2B, the template and drug appear to bind at different sites on the polymerase molecule. However, the binding of the template to the polymerase molecule appears to stabilize the enzyme against AF/ABDMP inhibition. Both AMV and M-MSV-(MLV) polymerase activity can be partially protected from inhibition when the template is preincubated with the enzyme (Table III). When the drug is added at high concentration during polymerization, the AMV polymerase is, for a short period of time, completely protected by the template; then a progressive inhibition is observed (Figure 3). At a concentration of 3.24×10^{-5} M drug, which completely inhibits enzyme activity when drug is added before template, the rate of reaction was not affected for 2 min and then decreased, approaching zero after 30 min (see insert). At a higher level of drug, 6.48×10^{-5} or 2×10^{-4} M (data not shown), the rate of reaction was unaffected for 60 sec, but then decreased, leveling off after 5 min. The effect of AF/ABDMP on the reverse transcriptase resembles, therefore, that of rifampicin on *E. coli* RNA polymerase (Lill *et al.*, 1970; So and Downey, 1970).

Effect of Increasing Enzyme Concentrations on the Initial Rate of Polymerization in the Presence of AF/ABDMP. Figure 4 illustrates the rate of DNA synthesis at different AMV polymerase concentrations in the presence of two different

TABLE III: Protection of DNA Polymerase Activity by Preincubation with Template.[a]

Polymerase	Preincubation with Template	Control [^{3}H]TTP Incorporated (cpm × 10^4)	[^{3}H]TTP Incorporated in the Presence of AF/ABDMP (cpm × 10^4)
AMV	−	16.9	4.4 (26)
	+	16.7	9.5 (57)
M-MSV(MLV)	−	5.3	0.8 (14)
	+	6.2	3.3 (53)

[a] AMV polymerase (1.35 μg) was preincubated (2 min total) in AMV diluting buffer (90 μl) with (+) or without (−) poly(A)-oligo(dT) at 25°. After a 1 min preincubation, Me_2SO (control) or AF/ABDMP was added. The reaction was initiated by adding the components of the standard reaction mixture with or without added template and aliquots were taken after 5, 15, and 30 min incubations at 37°. The final concentrations of polymerase and drug were 4.5 μg/ml and 2.4 × 10^{-5} M, respectively. The same experiment was repeated with M-MSV(MLV) polymerase (1 μg) in 60 μl of diluting buffer with or without poly(dA-dT) except the total preincubation time was 5 min. Me_2SO or AF/ABDMP were added after 2.5 min preincubation. The final polymerase and drug concentrations were 3.2 μg/ml and 2.2 × 10^{-5} M, respectively. The numbers in parentheses are expressed as a percentage of control activity.

concentrations of drug. At 1.4 × 10^{-5} M AF/ABDMP, the partial inhibition observed at the lower levels of enzyme was overcome when the enzyme concentration was increased. At 2.8 × 10^{-5} M drug, similar results were obtained. These data indicated a nonlinear relationship between enzyme concentration and AF/ABDMP inhibition of DNA synthesis and stimulated a more careful analysis of the relationship between the initial rate of polymerization and drug concentration.

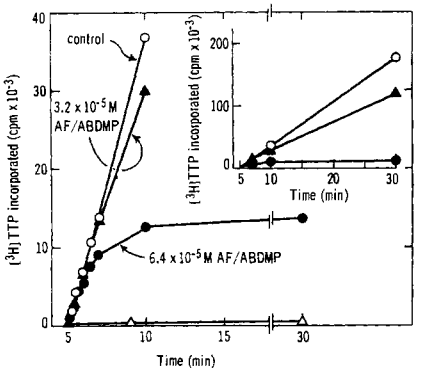

FIGURE 3: Protection by template of AMV DNA polymerase activity against inhibition by AF/ABDMP. AMV polymerase (5.8 μg/ml) was added to a standard reaction mixture containing poly(A)-oligo(dT) except that cold TTP (7 × 10^{-6} M) was present instead of [^{3}H]TTP. After 5 min incubation, [^{3}H]TTP (3 × 10^{-6} M) and AF/ABDMP at 3.2 (▲) or 6.4 (●) × 10^{-5} M were added together and aliquots taken as indicated. Only [^{3}H]TTP and Me_2SO were added to a control tube (○). In a separate experiment, the above enzyme was preincubated with AF/ABDMP (3.2 × 10^{-5} M) at 4° for 1 min with no template present in the above reaction mixture which had [^{3}H]TTP (3 × 10^{-6} M). Template was added at 0 min (Δ). A control without AF/ABDMP present (data not shown) demonstrated that the polymerase activity remained stable during preincubation. The insert presents data from the same experiment on an expanded scale.

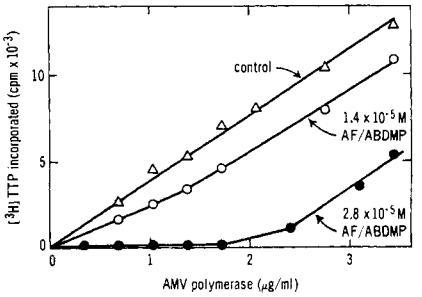

FIGURE 4: Effect of increasing AMV DNA polymerase concentration on the initial rate of polymerization measured at two different concentrations of AF/ABDMP. (A) Increasing concentrations of AMV polymerase were preincubated in 60 μl of AMV diluting buffer with either AF/ABDMP or Me_2SO for 1 min at 4°. The final enzyme concentration was varied as indicated and AF/ABDMP was at 1.4 × 10^{-5} M (○) and 2.8 × 10^{-5} M (●) with 2% Me_2SO (Δ) in the control. Each preincubation tube was initiated by the addition of prewarmed (37°) assay mixture (150 μl) containing poly(dA-dT). Aliquots (60 μl) were taken after 5, 10, and 15 min incubation, and initial velocities were plotted.

Effect of Varying AF/ABDMP Concentration on the Initial Rate of Polymerization Catalyzed by AMV DNA and E. coli RNA Polymerases. The rate of DNA and RNA synthesis was determined at different levels of drug using both AMV DNA and *E. coli* RNA polymerases, respectively. The AMV enzyme was directly added to the assay mixture without prior incubation with the drug. Under these conditions, relatively higher levels of drug are necessary to inhibit the enzyme. As shown in Figure 5, virtually no inhibition of DNA synthesis was obtained at concentrations below 2 × 10^{-5} M. Above this value inhibition was observed but the drug did not behave as a simple noncompetitive inhibitor, as indicated by the exponential behavior of the ratio V_0/V_i

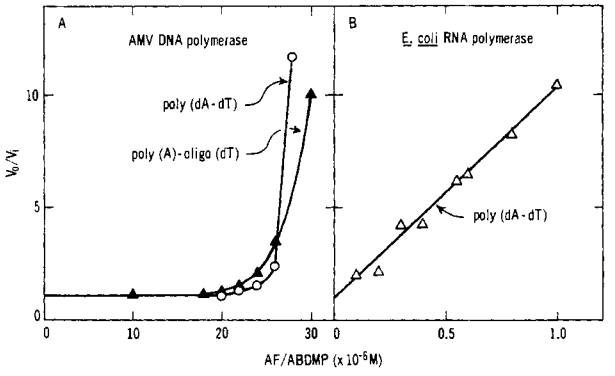

FIGURE 5: Cooperative interaction of AF/ABDMP molecules with AMV DNA polymerase. (A) Reaction mixtures containing poly(dA-dT) (○) and poly(A)-oligo(dT) (▲) were distributed into a separate series of tubes containing AF/ABDMP at the concentrations indicated. The reaction was initiated by AMV polymerase (1.2 μg/ml) and aliquots were taken from each tube at 1, 2, and 3 min of incubation. Using initial velocities, the ratio (V_0/V_i) (V_0 = [^{3}H]TTP incorporated in absence of drug; V_i = [^{3}H]TTP incorporated in presence of drug) was plotted as indicated. (B) *E. coli* RNA polymerase (12.5 μg) was preincubated 2 min at 4° in a series of tubes containing preincubation mixture (which lacked template and triphosphates) and AF/ABDMP at various concentrations. Tubes were then prewarmed for 20 sec at 37° and the reaction was initiated as described in Materials and Methods. After 30, 60, and 90 sec of incubation, aliquots were taken and initial velocities were used to calculate (V_0/V_i).

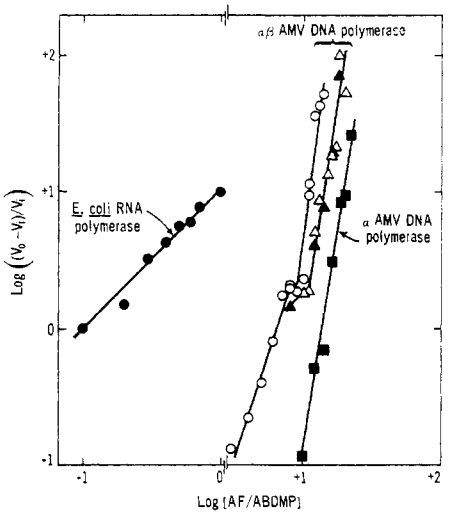

FIGURE 6: Plots of log $(V_0 - V_i/V_i)$ *vs.* log [AF/ABDMP] to determine Hill coefficients. V_0 is the initial velocity without AF/ABDMP and V_i is the initial velocity in the presence of AF/ABDMP. The Hill coefficient, n, is the slope of each line. Glycerol gradient purified $\alpha\beta$ AMV polymerase at 3.5 μg/ml was preincubated for 15 sec at 37° in 360 μl of assay mixture lacking template and primer, but containing 10 μCi (○) or 30 μCi (▲,△) of [^{3}H]TTP (10^{-4} M) and AF/ABDMP at the concentrations (μg/ml) indicated in the figure. The reaction was started by the addition of poly(A)-oligo(dT). After 2 min, 58,400 (△) and 59,000 cpm (▲) were incorporated in untreated controls, and in a third experiment, after 8 min, 90,000 cpm (○) were incorporated. Phosphocellulose purified α AMV polymerase at 15 μg/ml was assayed as described above (■); 15 μCi of [^{3}H]TTP (10^{-4} M) per assay were used. After 2 min, 40,000 cpm were incorporated in the untreated control. The data for *E. coli* RNA polymerase (●) included in this figure were obtained from the experiment illustrated in Figure 4. The molecular weight of the drug is 927.

(where V_i is the velocity in the presence and V_0 in the absence of AF/ABDMP) plotted as a function of AF/ABDMP concentration. Analogous results are obtained when the AMV enzyme is preincubated with drug before addition of template. This same type of inhibition has been observed with M-MSV-(MLV) DNA polymerase (data not shown). In contrast, when the inhibition of *E. coli* RNA polymerase by AF/ABDMP was studied under conditions similar to those used by Lill *et al.* (1970) to study the effect of rifampicin on the bacterial enzyme, a linear response between the ratio V_0/V_i and drug concentration was observed.

Cooperative Interaction among Binding Sites for AF/ABDMP on the AMV DNA Polymerase. The equilibrium equation for the reversible binding of AF/ABDMP to the enzyme is

$$\text{E-AF/ABDMP}_n/E[\text{AF/ABDMP}]^n = K$$

where E = drug free enzyme concentration, E-AF/ABDMP_n = drug–enzyme complex concentration, n = number of drug molecules bound per molecule of enzyme, and K = association constant. The above equation can be linearized in the form of the Hill equation which is used in studying the cooperative binding of ligands to proteins (Wyman, 1963). Therefore, we can evaluate the apparent number of molecules of AF/ABDMP that are needed to inhibit the AMV DNA polymerase

$$\log\left(\frac{\text{E-AF/ABDMP}_n}{E}\right) = \log\left(\frac{E^0 - E}{E}\right) = \log K + n \log [\text{AF/ABDMP}]$$

where $E^0 = E + \text{E-AF/ABDMP}_n$ = concentration of enzyme in the absence of drug. If we assume that the initial velocity measured in the presence of drug is due to free enzyme and that the binding of n molecules of AF/ABDMP per molecule of enzyme inhibit completely the polymerase activity, then the second equation becomes

$$\log\left(\frac{V_0 - V_i}{V_i}\right) = \log K + n \log [\text{AF/ABDMP}]$$

where V_0 = initial velocity in the absence of drug and V_i = initial velocity in the presence of drug.

The value for n can be obtained from the slope of the plot log $((V_0 - V_i)/V_i)$ *vs.* log [AF/ABDMP]. When the kinetic data obtained with $\alpha\beta$ polymerase, purified through glycerol gradient centrifugation, were plotted according to Hill, two values, 3 and 7, were observed for n with a change in the slope at about 60% inhibition, as illustrated in Figure 6. The experiments were performed by adding poly(A) and oligo(dT) to the assay mixture containing drug and enzyme after 15 sec preincubation. Similar results were found when the reaction was started by addition of enzyme without prior preincubation with AF/ABDMP, but as previously mentioned inhibition was obtained at relatively higher levels of drug. A high value for the Hill number (7 ± 0.5) above 40–50% inhibition was also obtained with phosphocellulose purified $\alpha\beta$ polymerase, with a decrease of the n value at lower drug concentrations. In contrast, a straight line was obtained with phosphocellulose purified α AMV polymerase with a value of 6.5 for n, which was constant over a range of drug concentration giving from 9 to 97% inhibition. A Hill coefficient of 1 was obtained when the inhibition of *E. coli* RNA polymerase by AF/ABDMP was analyzed in an analogous way. This result is in agreement with previous direct determination of the binding of the parental antibiotic rifampicin to the bacterial enzyme (di Mauro *et al.*, 1969).

Discussion

Several rifamycin derivatives have been found which inhibit the RNA directed DNA polymerase of RNA tumor viruses (see Riva and Silvestri, 1972, for a review). In this report we show that AF/ABDMP, closely related to rifampicin, inhibits the viral DNA polymerase by reversibly interacting with the enzyme itself and not with the template.

Like rifampicin, AF/ABDMP at very low concentrations inhibits *E. coli* DNA directed RNA polymerase. The bacterial RNA polymerase extracted from a rifampicin resistant strain is also resistant to AF/ABDMP, suggesting that rifampicin and AF/ABDMP inhibit the bacterial enzyme by the same mechanism (Szilagyi and Pennington, 1972). However, the mechanisms and the structural requirements for inhibition of the bacterial and viral polymerase are different: (1) the modification of the side chain of rifampicin is essential for inhibition of the viral DNA polymerase (Gurgo *et al.*, 1971); and (2) inhibition of the viral polymerase by AF/ABDMP is the result of the binding of several drug molecules, with cooperative interaction, while only one drug molecule binds per molecule of bacterial RNA polymerase (see Figure 6).

It is difficult to establish the number of drug molecules bound to the viral polymerase on the basis of kinetic data alone, for the Hill number is a function of the number of

binding sites and of the degree of interaction among these sites (Koshland, 1970). In addition, if the binding of drug causes dissociation of subunits, the Hill method would be inadequate for calculating the number of drug molecules bound, because the Hill equation does not take into consideration subunit dissociation. A minimum of six or seven molecules of drug is required for the inhibition of the α AMV DNA polymerase alone, which is a single polypeptide. High values for Hill numbers, although unusual, have been reported (Taketa and Pogell, 1965). Additional molecules of drug may bind in a nonspecific way without inhibiting the enzymatic activity. More difficult to understand is the change in the slope of the Hill plot in the case of the $\alpha\beta$ AMV DNA polymerase. It could suggest an additional alteration in the enzyme structure, perhaps an alteration in subunit pattern or conformation change. For example, the binding of drug molecules to α in the $\alpha\beta$ complex could cause its dissociation into α and β, with α being the only subunit with polymerase activity. The Hill number for α would be three in the $\alpha\beta$ complex, and seven for the free subunit if (1) β blocks some binding sites on α for the drug or (2) binding of β to α changes K_{app} of the sites for AF/ABDMP on α such that some are greater than others. Another interpretation is that binding of drug to $\alpha\beta$ causes dissociation of the two subunits, both having polymerase activity, with sequential inhibition of β and then α, since the value of n for the second slope is close to the value observed for the α subunit alone. However, this hypothesis requires that the β subunit have polymerase activity, which is not known, and a greater affinity for the drug than the α subunit.

Very likely the inhibition by AF/ABDMP of the viral polymerase is a consequence of a reversible structural modification of the enzyme; irreversible denaturation is excluded by the observation that dilution of drug promptly restores the enzymatic activity.

The fact that the drug does not compete with deoxyribonucleoside triphosphates or template for binding to the enzyme, and the lipophilic nature of the side chain may suggest that AF/ABDMP binds to hydrophobic regions removed from the active site of the enzyme. The finding that a low concentration of nonionic detergent may activate the reverse transcriptase (Thompson *et al.*, 1972) seems to suggest the possibility that hydrophobic regions on the viral polymerase may exert a regulatory function on the activity. Such a regulatory region would appear to be involved in the initiation of DNA synthesis, since the following lines of evidence implicate AF/ABDMP and a 3-cyclic amino derivative (Green *et al.*, 1972a) in the inhibition of the initial events of DNA synthesis: (a) preincubation of AMV polymerase with templates can partially protect against inhibition by the drug; (b) AF/ABDMP does not affect the K_m for polymerization of triphosphates; (c) there is no effect on the rate of TTP incorporation for at least 1 min when the drug is added at high levels during the reaction of polymerization. As in the case of inhibition of the bacterial polymerase by rifampicin (Sippel and Hartmann, 1970), the drug seems to block an early step in transcription. What this early step is, is completely unknown, for unlike the initiation of RNA synthesis by eucaryotic and bacterial RNA polymerase, which is inhibited by several rifamycins (Riva and Silvestri, 1972), and the initiation of RNA synthesis by T7 RNA polymerase, which is blocked by AF/ABDMP (Chamberlin and Ring, 1972), the initiation of DNA synthesis implies the addition of deoxyribonucleotides to an RNA primer. The details of this primer addition are only beginning to be studied.

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