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Different Modes of Inhibition of Purified Ribonucleic Acid Directed Deoxyribonucleic Acid Polymerase of Avian Myeloblastosis Virus by Rifamycin SV Derivatives†

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ABSTRACT: The mechanism by which several rifamycin SV derivatives inhibit the purified $\alpha\beta$ DNA polymerase of avian myeloblastosis virus was investigated. The derivatives C-27 (rifamycin SV with dicyclohexylalkyl-substituted piperidyl ring at the 3 position), AF-013 (*O-n*-octyloxime of 3-formylrifamycin SV), and AF/DNFI (3-(2,4-dinitrophenylhydrazonemethyl)rifamycin SV), with major structural differences in their hydrophobic side chains, were selected for study from among the most potent inhibitors. The effect of drug was studied under two different assay conditions: when drug was added before template-primer, in order to examine the inhibition of an initial step(s) of DNA synthesis, or during the reaction of polymerization, to examine the inhibition of elongation and/or reinitiation. The ability of C-27, AF-013, and AF/DNFI, in order of decreasing activity, to inhibit the viral DNA polymerase at an initial step(s) was directly related to the lipophilicity of the compounds. When inhibition of later steps was examined, no correlation was observed. C-27 was the least inhibitory of the three derivatives when added during polymerization; analysis of the mode of inhibition demonstrated that reinitiation, but not chain elongation, was inhibited. Incorporation of triphosphates into chains initiated prior to drug addition continued in the presence of C-27 and was progressively blocked at later times, while immediate, complete inhibition of triphosphate addition to new primer molecules followed drug addition. Polyacrylamide gel profiles of poly(dT) synthesized in the presence and absence of the drugs were compared. The amount of product synthesized in the presence of C-27 was decreased, but there was no effect on the size distribution. Both the amount and the size of the product were decreased in the presence of AF-013, suggesting an effect on chain elongation as well as initiation. Kinetic evidence indicated that AF/DNFI had a mode of action similar to that of AF-013. All three derivatives appear to inhibit the viral enzyme with a strong cooperative interaction. However, when the initial rate of polymerization measured at different drug concentrations was analyzed according to Hill, different plots were observed. A straight line with a slope of 6.4 was obtained in the presence of C-27, and a biphasic plot with n values of 2.2 and 6.2 was observed with AF/DNFI, with the change in slope occurring at 65% inhibition. The results of our study are discussed in terms of different mechanisms of interaction of rifamycin SV derivatives with the viral DNA polymerase.

The ability of several rifamycin derivatives to inhibit oncornavirus reverse transcriptase, and to various extents other DNA and RNA polymerizing enzymes, has been reported (Gurgo et al., 1971, 1972, 1975; Yang et al., 1972; Green et al., 1971, 1972; Meilhac et al., 1972; Chamberlin and Ring, 1972; Gerard et al., 1973; Rose et al., 1975) and attributed to the presence of a hydrophobic side chain in the basic structure of the parental antibiotic rifamycin SV. On the basis of this observation, more selective derivatives, able to distinguish between viral reverse transcriptase and other polymerases, have been synthesized (Tischler et al., 1973; Thompson et al., 1974).

Inhibition of cell transformation and/or oncornavirus replication by rifamycin derivatives has been reported by several laboratories (Barlati and Vigier, 1972; Hackett et al., 1972; Ting et al., 1972; Hackett and Sylvester, 1972; Green et al., 1972, 1974; Bissel et al., 1974; Smith and Hackett, 1974; Shannon et al., 1974; Szabo et al., 1976). Thus far, it appears that when cytotoxic effects are avoided the antiviral activity observed may be the result of the inhibition of the reverse transcriptase and/or interference with other postpenetrational viral functions. MuLV[1] replication and cell transformation by

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[1] Abbreviations used are: MuLV, murine leukemia virus; MSV, murine sarcoma virus; AMV, avian myeloblastosis virus; NP-40, Nonidet P-40; AF-013, *O-n*-octyloxime of 3-formylrifamycin SV; AF/DNFI, 3-(2,4-dinitrophenylhydrazonemethyl)rifamycin SV; AF/ABDMP, 2,5-dimethyl-4-*N*-benzyldemethylrifampicin SV; C-27, rifamycin SV with dicyclohexylalkyl substituted piperidyl ring at the 3 position; DEAE, diethylaminoethyl; EDTA, (ethylenedinitrilo)tetraacetic acid.

MSV were also inhibited by the parental compound, rifamycin SV, which lacks a side-chain component (O'Connor et al., 1974). It has been recently reported that this compound was able to inhibit MuLV reverse transcriptase by preventing the dissociation of the enzyme–template complex (Milavetz et al., 1976).

Studies on the mechanism of inhibition of the reverse transcriptase by selected derivatives indicated that the drugs bind to a site different from the active site, since AF/DNFI and AF/ABDMP do not compete for the binding of triphosphates or template, and that more than one molecule of drug binds per molecule of enzyme (Gurgo et al., 1974; Wu and Gallo, 1974). We reported that the inhibition appeared to be the result of a strong cooperative interaction among drug molecules which bind to a hydrophobic region of the reverse transcriptase (Gurgo et al., 1974). As previously found in the inhibition of bacterial (Riva and Silvestri, 1972, for a review), T7 bacteriophage-induced (Chamberlin and Ring, 1972), and mammalian RNA polymerases (Meilhac et al., 1972, and Tsai and Saunders, 1973) by rifamycin derivatives, these compounds are more effective when they interact with the reverse transcriptase before initiation of polymerization (Wu and Gallo, 1974). In this article, we present evidence that the differences in the structure of the side chain confer on several rifamycin derivatives the ability to inhibit the viral DNA polymerase with different modes of action.

Materials and Methods

Materials. The rifamycin derivatives AF-013, AF/DNFI, and AF/ABDMP were a generous gift from Prof. G. Lancini of Gruppo Lepetit, Milan, Italy; C-27 was kindly provided by Dr. H. Heymann of the Pharmaceuticals Division of CIBA-GEIGY Corp., Summit, N.J. Their respective molecular weights are: 853, 905, 929, and 959. The drugs were dissolved in Me_2SO at 10 mg/ml and stored at 4 °C. Urea (ultrapure grade) was from Schwarz/Mann; acrylamide and bisacrylamide were purchased from Bio-Rad; silicone 350 cs was from Dow Corning; thin-layer chromatography plates, 20 × 20 cm, 250-μm thick, precoated with silica gel, were made by Analtech, Inc.; oligo(dT)$_{(12-18)}$ and oligo(dG)$_{(12-18)}$ were from Collaborative Research; poly(A) and poly(C) were from Miles Laboratories; unlabeled deoxyribonucleoside triphosphates were from P-L Biochemicals; [^{3}H]TTP, [^{3}H]dGTP, and [^{32}P]TTP were from New England Nuclear Corp.; DEAE-cellulose disks (DE-81) were from Reeve Angel.

Virus. AMV was generously provided by Dr. Joseph Beard, Life Sciences, Inc., through the Office of Program Resource and Logistics of the Virus Cancer Program. The virus was purified as previously described (Green et al., 1970). The major DNA polymerase species isolated from AMV, designated $\alpha\beta$, was purified from NP-40 detergent lysed virus, using ammonium sulfate precipitation, followed by DEAE-cellulose and phosphocellulose chromatography (Grandgenett et al., 1973). The enzyme was further purified on hydroxylapatite, in the absence of NP-40, according to Kacian et al. (1971). The $\alpha\beta$ DNA polymerase was 85% pure, as judged by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The enzyme was stored at −20 °C in 0.2 M potassium phosphate buffer, pH 8, 5 mM dithiothreitol, 0.1 mM EDTA, and 50% glycerol. It was diluted with the same buffer, minus EDTA, with 10% glycerol prior to use.

DNA Polymerase Assay. The assay mixture for the synthesis of poly(dT) contained: 45 mM Tris-HCl, pH 8.3, 30 mM NaCl, 4 mM $MgCl_2$, 0.1 mM each of dATP, dCTP, dGTP, 0.1 or 0.01 mM [^{3}H]TTP (10–30 μCi/assay), 3 mM dithiothreitol, 20 μg/ml of poly(A), and 1 μg/ml of oligo(dT), in a final volume of 0.1 ml. For poly(dG) synthesis, poly(C) and oligo(dG) were used at 20 and 1 μg/ml, respectively, with [^{3}H]dGTP as labeled substrate. All four triphosphates were present in the standard assay mixture in order to render the assay conditions comparable to those used in previous work (Gurgo et al., 1974). For studying the effect of drug on an initial step(s) of DNA synthesis, the reaction was initiated by the addition of template-primer, as described above, to the assay mixture 20 s after the addition of enzyme to the same mixture, preincubated at 37 °C, containing drug or Me_2SO. The TTP concentration in these experiments was 0.1 mM. For studying the effect of drug on step(s) subsequent to initiation, the reaction was started by addition of template-primer to the assay mixture containing unlabeled TTP. Labeled TTP was subsequently added, together with drug or Me_2SO, or shortly thereafter, as indicated. The final concentration of TTP was 0.01 mM. The reactions were terminated by the addition of EDTA, and the amount of radioactive product formed was determined on DEAE-cellulose filters (Blatti et al., 1970).

Size Fractionation of Poly(A) Template by Gel Chromatography. Large size poly(A) was isolated from commercially available preparations in order to obtain a relatively uniform template for DNA synthesis. Poly(A) (100 μmol PO_4/ml) was dissolved in buffer containing 20 mM Tris-HCl, pH 8, 0.1 M NaCl, and 6 M urea. One-milliliter aliquots were filtered through a Sephadex G-200 column (1.5 × 90 cm) equilibrated with the same buffer. One-milliliter fractions were collected, and the elution of poly(A) was monitored by measuring the absorbance at 260 nm. Enrichment for higher molecular weight poly(A) was obtained by pooling the first 10 fractions of the eluted sample from each chromatographic run; this pool was approximately 15% of the total material loaded on the column. The pool was dialyzed free of urea and NaCl was added to a final concentration of 0.2 M, followed by two volumes of absolute alcohol. After 12 h at −20 °C the precipitated poly(A) was collected by centrifugation, and the pellet was resuspended in 0.1 M NaCl with 20 mM Tris-HCl, pH 8, and then dialyzed against the same buffer.

Polyacrylamide Gel Electrophoresis of Poly(dT) Synthesized by $\alpha\beta$ DNA Polymerase. The poly(dT) product synthesized in the presence and absence of drug was purified and analyzed by gel electrophoresis in order to detect any effect of drug on its size. Equal aliquots from reaction mixtures containing poly(dT) product synthesized in the presence (^{32}P-labeled) and absence (^{3}H-labeled) of drug were mixed, incubated overnight at 37 °C with 0.2 M NaOH, neutralized with HCl, and passed over a Sephadex G-50 column (1.2 × 60 cm) equilibrated with 0.02 M ammonium bicarbonate in order to separate the product from labeled triphosphates. Fractions were collected and aliquots from each were counted in a liquid scintillation system to monitor the elution position of the poly(dT) product. Fractions containing the labeled product were pooled, lyophilized, and resuspended in distilled water.

Ten percent polyacrylamide gels (0.5 × 13 cm) with 7 M urea were used for product-size analysis (Grandgenett and Green, 1974). Approximately 80-μl samples containing given amounts of poly(dT) product, 20% sucrose, 7 M urea, and bromphenol blue were layered on top of a 1-cm spacer. A constant current of 5 mA/gel was applied. The length of the run was adjusted to allow the maximal migration of a 5′-end labeled [^{32}P]oligo(dT)$_{(12-18)}$ marker. Gels were fractionated into 2-mm slices with a Gilson automatic gel fractionator, dissolved in 10 ml of Aquasol, and counted.

Determination of the R_M of Rifamycin Derivatives. Glass

TABLE I: Lipophilicity and Inhibitory Activity of Rifamycin Derivatives.

Derivative	R_M[a]	EC_{50}[b] (initiation) (M)	% Inhibition (elongation and/or reinitiation)
C-27	0.76	1.7×10^{-6}	53
AF-013	0.33	5.2×10^{-6}	98
AF/DNFI	−0.16	1.16×10^{-5}	92
AF/ABDMP	−0.03	2.08×10^{-5}	50

[a] $R_M = \log (1/R_F - 1)$; the R_F value of each compound was measured as described under Materials and Methods. The R_M values are the average of six determinations. [b] EC_{50} is the effective drug concentration which gives 50% inhibition of $\alpha\beta$ DNA polymerase activity when the enzyme is added to the assay mixture containing drug, prior to addition of poly(A)·oligo(dT). The EC_{50} values were obtained from Hill plots, as shown in Figure 7. The EC_{50} for C-27 was the average of six determinations with drug concentrations varying from 1.4 to 2 μg/ml; EC_{50} for the other derivatives were the average of two determinations. The side-chain components of AF-013 and AF/ABDMP were inactive at 200 μg/ml, the highest dose tested. The percent inhibition of elongation and/or reinitiation was calculated by measuring the radioactivity incorporated after 10 min of incubation, with 60 μg/ml of drug added during the reaction of polymerization, as indicated in the legend to Figure 1B. This corresponds to a concentration of 6.2, 7.0, 6.3, and 6.4×10^{-5} M for C-27, AF-013, AF/DNFI, and AF/ABDMP, respectively. $\alpha\beta$ DNA polymerase was used at a final concentration of 1.28 μg/ml.

plates precoated with silica gel G were impregnated with 5% silicone in ether to obtain a stationary nonpolar phase, according to the method of Biagi et al. (1969). Drug samples were dissolved in acetone at a final concentration of 1 mg/ml. Two-microliter aliquots were applied to the plate at 1.5-cm intervals. The plates were developed in a chromatography tank containing different concentrations of acetone–water saturated with silicone. The solvent front was allowed to migrate 10 cm. The R_M values were calculated as described by Pelizza et al. (1973).

Results

Lipophilicity and Inhibitory Effects of Rifamycin Derivatives. The degree of lipophilicity of the rifamycin derivatives used in this study was measured by determining the value of the chromatographic parameter R_M, which has been shown to be related to the logarithm of the partition coefficient (Bate-Smith and Westall, 1950). This parameter has been used for correlating structure and biological activity of lipophilic compounds, such as penicillins and rifamycins (Biagi et al., 1969; Pelizza et al., 1973). R_M was calculated according to the following equation: $R_M = \log (1/R_F - 1)$. The R_F values for each drug were determined on reverse thin-layer chromatography on silica gel plates using as a mobile phase various mixtures of acetone and water (Boyce and Milborrow, 1965). In Table I, R_M values are reported together with the drug concentrations giving 50% inhibition of $\alpha\beta$ DNA polymerase activity (EC_{50}). The EC_{50} values were obtained from Hill plots of kinetic data (as in Figure 7) and represent inhibition of enzymatic activity prior to chain elongation. An inverse relationship was found between R_M and EC_{50}. As the value of R_M decreased, the concentration of the particular drug giving 50% inhibition increased. Such a correlation has been observed with cyclic and acyclic rifamycin derivatives of two analogous series (Tischler et al., 1974).

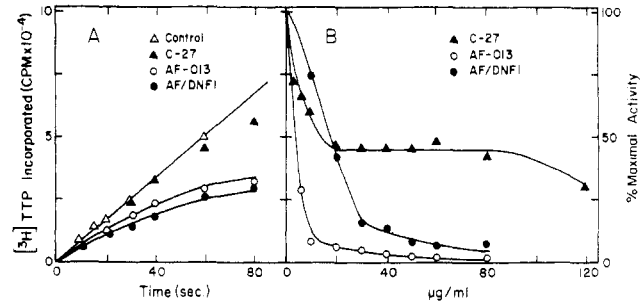

FIGURE 1: Effect of drug addition during the reaction of polymerization. (A) $\alpha\beta$ DNA polymerase (1.28 μg/ml) was added to a standard assay mixture containing unlabeled TTP. After 2-min incubation at 37 °C, labeled TTP and Me_2SO or drug (60 μg/ml) were added. Ninety-microliter aliquots were removed at the indicated times and radioactivity incorporated into poly(dT) was determined as indicated under Materials and Methods. (B) Labeled TTP and different amounts of drug were added to several assay mixtures under the conditions described in panel A. The activity measured after 10-min incubation is shown as a function of drug concentration; 51 700 cpm were incorporated in the uninhibited control.

Inhibition of steps subsequent to chain initiation, i.e., chain elongation and/or reinitiation, was then measured by adding drug to a DNA-synthesizing reaction. Due to the size heterogeneity of the polymers synthesized in the system using poly(A)·oligo(dT) as template-primer and the impossibility of synchronizing the synthesis of discrete size classes of poly(dT), an arbitrary time for drug addition, 2 min after the reaction was initiated by template-primer addition, was chosen. Incorporation was then followed for 10 min to allow completion of large transcriptional units initiated prior to drug addition. As seen from the comparison of the EC_{50} and the concentration of drug used to inhibit the enzyme during the ongoing reaction of polymerization, all three drugs appeared to be less effective under these assay conditions (Table I). No correlation between lipophilicity and inhibition of enzymatic activity was observed. C-27, the most effective inhibitor of an initial step(s) of DNA synthesis, was less effective than AF/DNFI or AF-013 when added to the polymerizing system (Table I).

The inference that C-27 inhibits the $\alpha\beta$ DNA polymerase by a mechanism different from that of the other compounds was strengthened by the observation that the rate of polymerization was unaffected for about 1 min upon addition of C-27, while inhibition following the addition of the same amount of AF-013 or AF/DNFI occurred even at very short times (Figure 1A). Furthermore, when incorporation of [^{3}H]TTP following drug addition was monitored at various drug concentrations at a given time, the percent inhibition was dose dependent for AF/DNFI and AF-013, but not for C-27 (Figure 1B). After 10-min incorporation in the presence of drug, about 90% inhibition was measured with AF/DNFI and AF-013 at concentrations two and four times their respective EC_{50}. Under the same conditions, a constant value of 55% inhibition was obtained with C-27 concentrations 10–50 times its EC_{50}. These results suggest that $\alpha\beta$ DNA polymerase can reinitiate in the presence of low doses of C-27 (Figure 1B); with increasing drug concentrations, reinitiation is progressively inhibited, while elongation remains unaffected over a discrete range of concentrations. Both reinitiation and elongation appear to be inhibited by AF-013 and AF/DNFI.

The order in which drug and enzyme interact affects not only the efficiency of inhibition (Gurgo et al., 1974, and this publication; Wu and Gallo, 1974), but also the linearity of DNA synthesis. When the enzyme interacts with a limiting amount of drug before addition of template-primer, the rate

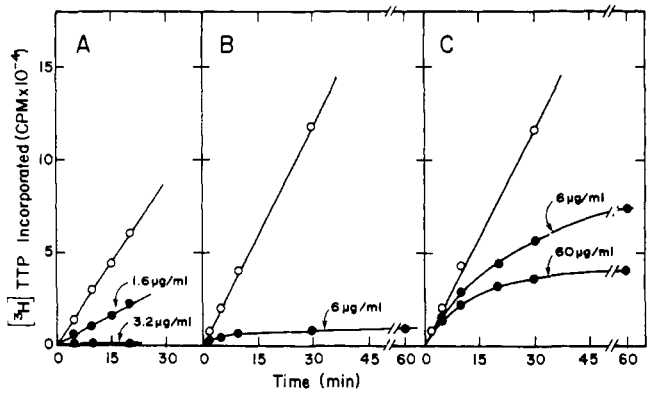

FIGURE 2: Effect of the order of addition of enzyme, drug, and TTP on the rate of polymerization. (A) $\alpha\beta$ DNA polymerase was added to a standard assay mixture containing Me_2SO (○) or C-27 (●), at the indicated concentration, but lacking poly(A)·oligo(dT). After 20-s incubation at 37 °C, poly(A)·oligo(dT) was added to start the reaction. DNA synthesis was followed as indicated. (B) DNA synthesis was initiated by the addition of $\alpha\beta$ DNA polymerase to an assay mixture containing poly(A)·oligo(dT) and Me_2SO (○) or C-27 (●). Aliquots were removed at given times and the amount of [^{3}H]TTP incorporated was determined. (C) The assay for addition of drug during polymerization was as described in the legend to Figure 1A. In A, B, and C, the final concentration of [^{3}H]TTP and $\alpha\beta$ DNA polymerase was 1×10^{-4} M and 1.28 μg/ml, respectively.

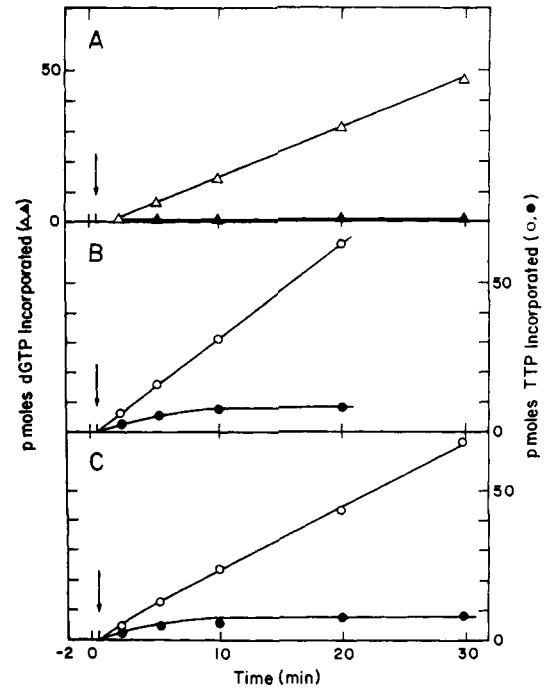

FIGURE 3: Effect of C-27 on chain reinitiation. (A) $\alpha\beta$ DNA polymerase (1.6 μg/ml) was added to an assay mixture (800 μl) containing 0.9×10^{-5} M unlabeled TTP, 1.2×10^{-5} M [^{3}H]dGTP, and poly(A)·oligo(dT). After 2 min of DNA synthesis at 37 °C, the mixture was divided into two aliquots and C-27 (▲) or Me_2SO (△) was added. After 20 s (see arrow), poly(dG) synthesis was initiated by addition of poly(C)·oligo(dG) (40 and 2 μg/ml, respectively, a twofold excess over poly(A)·oligo(dT)) along with unlabeled TTP in 100 μl. (B) The $\alpha\beta$ DNA polymerase was added to an identical assay mixture for poly(dT) synthesis, as described in A. After 2-min incubation, C-27 (●) or Me_2SO (○) was added, followed 20 s later (see arrow) by the addition of [^{3}H]TTP and a twofold excess of poly(A)·oligo(dT) over the amount already present. (C) $\alpha\beta$ DNA polymerase was added to an assay mixture containing poly(A)·oligo(dT) as described in A, but containing unlabeled dGTP (1.2×10^{-5} M). Incorporation of [^{3}H]TTP was monitored after addition of labeled TTP (see arrow). Synthesis of poly(dG) was allowed to proceed by the simultaneous addition of poly(C)·oligo(dG) (40 and 2 μg, respectively). The final concentration of C-27 was 60 μg/ml; that of TTP and dGTP was 1×10^{-5} M in panels A, B, and C.

of the reaction of polymerization is linear, as illustrated in Figure 2A. It appears that the effect of the drug is to reduce the number of enzyme molecules free to initiate polymerization. When the same amount of enzyme is allowed to interact with the drug at a concentration sufficient to give complete inhibition, but in the presence of template-primer as illustrated in Figure 2B, limited DNA synthesis is observed. Very likely, a fraction of enzyme molecules becomes resistant as a result of binding to the template-primer. The rate of the inhibited reaction is not linear, suggesting that the enzyme molecules are progressively inhibited upon chain termination. Similarly, the rate of deoxyribonucleotide incorporation decreases with time when the drug is added during the polymerization reaction; higher levels of drug are necessary in order to obtain inhibition (Figure 2C), since all enzyme molecules are in their most resistant form, that is, engaged in the process of chain elongation at the time of the addition.

Inhibition of Initiation and/or Chain Elongation. To more firmly establish that C-27 inhibits chain reinitiation while allowing elongation to proceed, the effect of the drug on the addition of triphosphates to primer molecules was investigated, using a level of drug in the range of concentrations in which the percent inhibition remained constant (Figure 1B). In order to distinguish between incorporation of triphosphates into chains initiated before drug addition and elongated in the presence of drug from incorporation of triphosphates into chains initiated after C-27 addition, two different template-primer systems were used, poly(A)·oligo(dT) and poly(C)·oligo(dG). Synthesis of the corresponding polymers was allowed to proceed in the same assay. Poly(dT) synthesis was always initiated before addition of drug or Me_2SO, and poly(dG) synthesis, after. The synthesis of the product directed by one or the other template-primer combination was monitored in parallel experiments in which one of the two substrate triphosphates, TTP or dGTP, was labeled. Such an experiment is shown in Figure 3. Polymerization of unlabeled TTP into poly(dT) was allowed to proceed for 2 min (Figure 3A), the assay mixture was then divided into two aliquots to which C-27 or Me_2SO was added, followed 20 s later by the addition of poly(C)·oligo(dG) to both aliquots. Unlabeled TTP was also added to bring the concentration to the value used in the standard assay mixture. Incorporation of [^{3}H]dGTP present in the original assay mixture was observed after a short lag following addition of poly(C)·oligo(dG) to the Me_2SO-treated sample, while no incorporation was detected in the presence of drug (Figure 3A). In a control experiment (Figure 3B), polymerization of TTP was revealed by the addition of [^{3}H]TTP to the Me_2SO- and C-27-containing mixtures. Only polymerization of TTP occurred, since poly(A)·oligo(dT), instead of poly(C)·oligo(dG), was added together with [^{3}H]TTP. In this case, some polymerization of TTP occurred in the presence of the drug. In a second control experiment (Figure 3C), polymerization of labeled TTP was allowed to occur together with the simultaneous polymerization of unlabeled dGTP, present in the original assay mixture. After addition of drug or Me_2SO, labeled TTP and poly(C)·oligo(dG) were added to the two aliquots. Polymerization of [^{3}H]TTP in the Me_2SO-treated control proceeded at a lower rate following addition of template-primer, indicating engagement of some polymerase molecules in the synthesis of unlabeled poly(dG). Incorporation of [^{3}H]TTP in the presence of C-27 occurred as in Figure 3B.

Effect of Drug Addition on the Product Size. The effect of

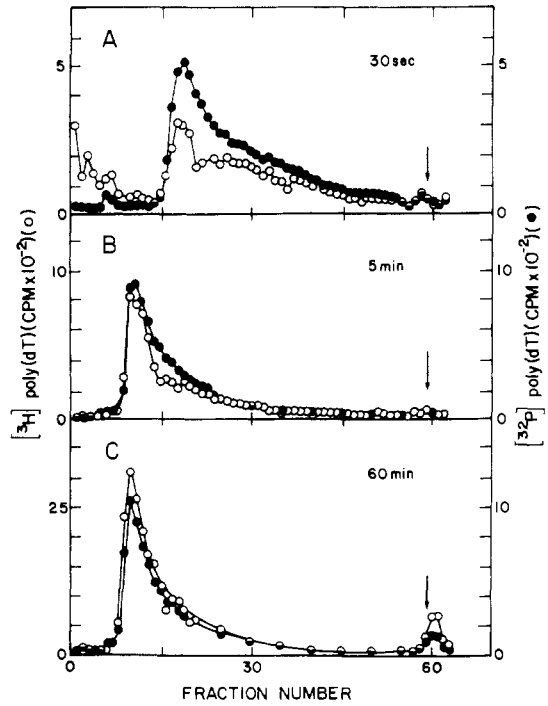

FIGURE 4: Effect of C-27 on the size of the poly(dT) synthesized. $\alpha\beta$ DNA polymerase was added to an assay mixture containing unlabeled TTP and poly(A)·oligo(dT). After 2-min incubation, two aliquots of 900 μl were transferred to prewarmed tubes, and the volume was brought to 1.2 ml by the addition of [^{3}H]TTP and Me_2SO (○) or [^{32}P]TTP and C-27 (●). The final concentrations of $\alpha\beta$ DNA polymerase, C-27, and TTP were 1.28 μg/ml, 60 μg/ml, and 1×10^{-5} M, respectively. At the indicated times, aliquots were removed from each incubation mixture and the reaction was stopped with EDTA. ^{3}H- and ^{32}P-labeled poly(dT) from each respective time point were mixed, treated with NaOH, neutralized, and purified as indicated under Materials and Methods. The size distributions of the products after coelectrophoresis on polyacrylamide gels are illustrated in panel A, 30 s; B, 5 min; and C, 60 min. The arrow indicates the position of a [^{32}P]oligo(dT) marker. The specific activities of [^{3}H]TTP and [^{32}P]TTP were 22.5 and 8.3 Ci/mmol, respectively.

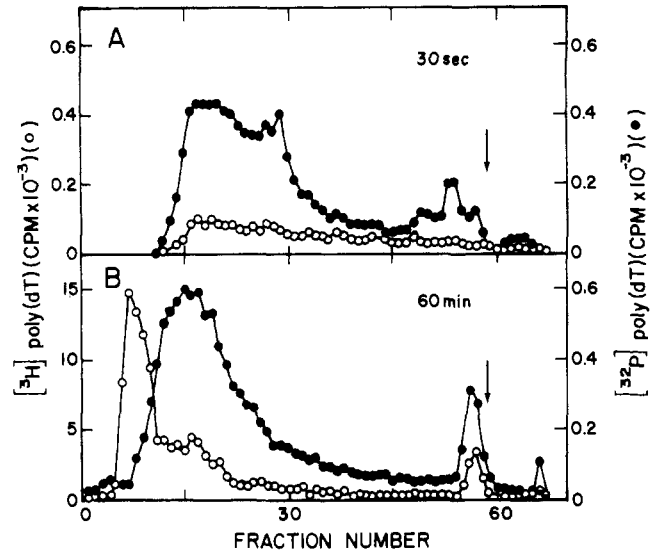

FIGURE 5: Effect of AF-013 on the size of the poly(dT) synthesized. The experimental conditions were as described in the legend to Figure 4; AF-013 was used at 60 μg/ml. Aliquots were taken at 30 s (A) and 60 min (B) and subjected to electrophoresis.

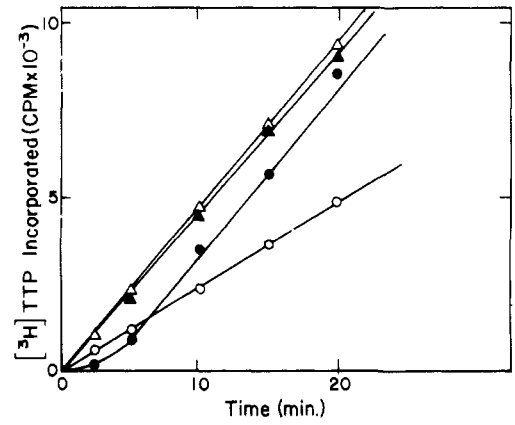

FIGURE 6: Reversibility of drug inhibition. $\alpha\beta$ DNA polymerase (1.28 μg/ml) was added to two assay mixtures containing Me_2SO or drug. Twenty seconds later, poly(A)·oligo(dT) was added to initiate DNA synthesis. One minute later, an aliquot was removed and diluted tenfold with prewarmed standard assay mixture containing 1 mg/ml of bovine serum albumin. At the indicated times, aliquots were removed from the diluted and undiluted assay mixtures, and the incorporated radioactivity was counted. (△) control, (▲) AF/DNFI, (○) AF-013, (●) C-27. The drug concentrations in the undiluted assay mixtures were 40, 8, and 8 μg/ml, respectively; at these concentrations, the enzymatic activity was completely inhibited.

addition of C-27 and AF-013 during DNA synthesis on the size of poly(dT) product was studied. At the ratio of poly(A) to oligo(dT) used throughout this study (20/1), about 1 molecule of primer per molecule of template is present in the assay. However, due to some heterogeneity in the size of the poly(A) template and the random binding of oligo(dT) to poly(A), a distribution of poly(dT) product is expected, with sizes ranging between that of the primer and the largest template molecule. Any drug effect on chain elongation should be detected by comparing the pattern of distribution of the product in the presence and absence of drug. As shown in Figure 4B, C, 5 min after the addition of [^{3}H]TTP to the system synthesizing unlabeled TTP, all size classes of poly(dT) are represented, since the gel patterns at 5 and 60 min are identical. The entrance of ^{32}P-labeled TTP into poly(dT) chains initiated prior to the addition of C-27 is not inhibited by the drug, since after 60 min of incubation, when no further incorporation is observed, the profile of the ^{32}P-labeled product is identical to that of the ^{3}H-labeled poly(dT) control, the only difference being the amount of the product made. In contrast, as expected from kinetic data, both the size and the amount of poly(dT) synthesized in the presence of AF-013 are affected (Figure 5A,B).

Reversibility and Multiplicity of Binding Sites for Rifamycin Derivatives. An previously described for AF/ABDMP (Gurgo et al., 1974), C-27 and AF/DNFI bind reversibly to the reverse transcriptase, as demonstrated by the full recovery of activity after dilution and competition with excess bovine serum albumin (Figure 6). In the case of C-27, the complete recovery of activity is not immediately obtained upon dilution, and a lag is observed before the rate of polymerization assumes the same value as the uninhibited control. Only 50% of the activity inhibited by AF-013 was recovered, indicating a tighter binding for this derivative (Figure 6). Reversibility of the binding of AF/DNFI and AF/ABDMP has also been obtained by treatment of the inhibited enzyme with nonionic detergents (Wu and Gallo, 1974).

The effect of AF/DNFI and C-27 on the initial rate of polymerization was studied at different drug concentrations. As previously noted for AF/ABDMP, the inhibition of the $\alpha\beta$ DNA polymerase appears to be the result of the binding of

more than 1 molecule of drug per molecule of enzyme with a cooperative interaction among the ligand molecules. In order to estimate the number of interacting binding sites for the inhibitor molecules, the velocity data were analyzed according to the Hill equation (Taketa and Pogell, 1965; Koshland, 1970), assuming that the fraction of the total number of binding sites occupied by the drug is proportional to the ratio V_i/V_0 (where V_i and V_0 are the initial velocities measured in the presence and absence of drug) throughout the range of drug concentrations affecting enzymatic activity. When the inhibition data obtained with the derivative AF/DNFI were plotted, a biphasic plot was observed with a slope of 2.2 in the range of drug concentrations which give from 10 to 65% inhibition and a slope of 6.2 above this range. A similar pattern was observed with the derivative AF/ABDMP (Gurgo et al., 1974). A straight line with a slope of 6.4 was obtained when the same analysis was extended to the inhibitory effect of C-27 on the viral enzyme. Assuming that the binding is highly cooperative, a minimum of three molecules of AF/DNFI appear to bind per molecule of enzyme. The change in slope suggests that an additional alteration in the enzyme structure occurs above drug concentrations giving 65% inhibition such that a higher number of molecules bind, approximately 7. Such alteration appears to occur over the entire range of data with the more hydrophobic derivative C-27, since a straight line with an angular coefficient of 6.4 is observed.

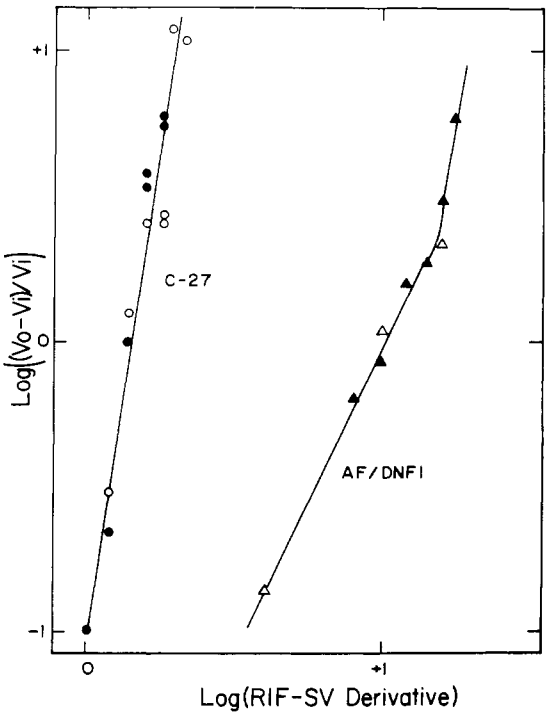

FIGURE 7: Plot of log $(V_0 - V_i)/V_i)$ vs. log of the drug concentration, for calculation of the Hill number. $\alpha\beta$ DNA polymerase was added to an assay mixture, lacking poly(A)·oligo(dT), and containing various concentrations of drug, as indicated. After 20-s preincubation at 37 °C, the reaction was initiated by the addition of poly(A)·oligo(dT), and aliquots were removed after 5, 10, and 15 min. V_0 is the initial velocity without drug, and V_i is the initial velocity in the presence of drug. Each plot is the result of two different experiments. A slope of 6.4 was obtained in the case of C-27; a slope of 2.2 was obtained with AF/DNFI, with a change to approximately 6.2 at 65% inhibition. After 19 min, 23 000 (○), 29 000 (●), 21 000 (△), and 50 000 (▲) cpm were incorporated in the untreated control.

Discussion

The objective of the work presented in this article was to investigate the mechanism of inhibition of the reverse transcriptase by different rifamycin derivatives in order to evaluate the possibility of a basic mechanism common to this class of compounds. The three derivatives selected for study are from among the most potent inhibitors of the reverse transcriptase and have major differences in the structure of their side chain.

DNA synthesis directed by a synthetic template-primer system can be hypothesized to consist of the following steps: (1) binding of enzyme to template-primer (preinitiation), (2) formation of the first phosphodiester bond between a deoxynucleotide triphosphate molecule and a primer molecule (initiation), and (3) chain elongation. Previous studies indicated that binding of enzyme to template-primer confers on the enzyme molecules variable degrees of protection against inhibition by rifamycin compounds; a more stable complex is formed during the polymerization reaction (Wu and Gallo, 1974; Gurgo et al., 1974). This appears to be the only similarity with the mechanism by which the bacterial RNA polymerase is inhibited by rifampicin (Sippel and Hartman, 1970; So and Downey, 1970; Hinkle et al., 1972). The most evident difference, in addition to a lower affinity, is the multiplicity of the drug molecules binding to the viral enzyme, as demonstrated for the derivatives AF/ABDMP and AF-013. Direct determination of the binding of rifamycin derivatives to the reverse transcriptase by glycerol-gradient centrifugation of a mixture of labeled drug and enzyme provided variable ratios of 2–14 molecules of [^{14}C]AF/ABDMP bound per molecule of MSV DNA polymerase (Wu and Gallo, 1974). At least two molecules interact with a tight and more specific bond. The observed variability suggests that some molecules bind with a low affinity and in a nonspecific way without necessarily contributing to the inhibition of enzymatic activity.

In the previous study on the mechanism of inhibition of the reverse transcriptase by the derivative AF/ABDMP, we observed that small variations in drug concentrations produced marked effects on enzymatic activity. We then presented kinetic evidence which indicated cooperative interaction among the binding sites for the rifamycin derivative AF/ABDMP (Green et al., 1974; Gurgo et al., 1974). In this article, we report similar findings for the derivatives C-27 and AF/DNFI. Working hypotheses for the interpretation of the observed Hill plots were presented in the previous article.

The rifamycin derivatives which strongly inhibit the viral reverse transcriptase have in common the hydrophobic character of the side-chain components, which are inactive when separated from the basic chromophore moiety (Gurgo et al., 1971; this report). If, however, the ability to inhibit were only the consequence of lipophilicity, then a common, nonspecific mechanism should be implied, the only difference being in the level of the effective dose. The results of the studies on the mechanism of action of C-27, AF-013, and AF/DNFI presented in this article do not support this hypothesis and indicate different modes of action.

The correlation between lipophilicity and the ability to inhibit the reverse transcriptase (Tischler et al., 1974; this report) is observed only when the drug–enzyme interaction occurs prior to initiation of DNA synthesis. When the drug target is the enzyme engaged in the process of chain elongation, other factors, probably structure related, appear to be relevant to the mechanism of interaction. C-27, the most hydrophobic and the most effective inhibitor of the derivatives studied when added before initiation of polymerization, is the least inhibitory when added during polymerization. We have shown that C-27 is a

selective inhibitor of an initial step of transcription, since the size of the DNA product synthesized during the reaction is not affected by the drug when added during synthesis at doses 50–60 times the EC_{50}; only reinitiation appears to be inhibited under these conditions. Using another approach which involved template immobilized in agarose, Milavetz et al. (1976) have recently reported that rifamycin SV, AF/ABDMP, AF-013, and other related compounds inhibit MuLV reverse transcriptase with different mechanisms. Thus, the evidence presented so far strongly indicates the influence of structural differences in addition to lipophilicity on the selective inhibition of specific steps of transcription by different rifamycin SV derivatives.

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