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Action of Rifamycin Derivatives on RNA Polymerase of Human Leukemic Lymphocytes

(AF/013)(PR/19)

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ABSTRACT **The mechanism of inhibition of human RNA polymerase by four rifamycin derivatives was investigated. Derivative AF/013 (3-formyl rifamycin SV:*O*-*n*-octyloxime) with strong hydrophobic side chains prevents the polymerase from binding to DNA and also affects the size of RNA synthesized. Derivative PR/19 (3′-acetyl-1′-benzyl-2′-methylpyrrolo[3,2-c]-4-desoxy-rifamycin SV) only affects RNA synthesis when RNA polymerase has been previously incubated with the drug or when the reaction was performed at high salt concentration [0.14 M $(NH_4)_2SO_4$]. Our results suggest that these drugs exert their inhibitory actions by binding to the enzyme instead of DNA.**

The antibiotic rifampicin inhibits DNA-dependent RNA polymerase obtained from prokaryotic cells, but it has no effect on RNA polymerase isolated from higher organisms (1). One rifamycin derivative, AF/013 (3-formyl rifamycin SV:*O*-*n*-octyloxime) synthesized by Lepetit S.p.A., Milan, Italy, can inhibit DNA-dependent RNA polymerases isolated from rat liver (2) and calf thymus (3). This drug inhibits initiation of RNA chains (2, 3). We report studies of the action of rifampicin and four rifamycin derivatives: AF/05 (3-formyl rifamycin SV:*O*-[diphenylmethyl] oxime), AF/013, PR/19 (3′-acetyl-1′-benzyl-2′-methylpyrrolo[2,3-c]-4-desoxy-rifamycin SV), and PR/14 (3′-acetyl-1′, 2′-dimethyl-pyrrolo[3,2-c]-4-desoxy-rifamycin SV) on DNA-dependent RNA polymerase II (or B) isolated from lymphocytes of patients with chronic lymphocytic leukemia.

In vitro RNA synthesis by DNA-dependent RNA polymerase proceeds in a stepwise manner (3, 4). The first step involves binding of the enzyme to the DNA template; the subsequent steps are chain initiation, chain elongation, and chain termination. Rifampicin inhibits *Escherichia coli* RNA polymerase after binding of polymerase to the DNA and before formation of the first internucleotide bonds (1, 5, 6). Thus, once RNA synthesis has been initiated, rifampicin does not inhibit further elongation of the RNA chains.

The results presented here indicate that rifamycin derivatives AF/05, AF/103, and PR/19 inhibit initiation of RNA chains by binding to the polymerase, thus preventing the enzyme from binding to DNA. Furthermore, we find that AF/013 and AF/05 can inhibit and can cause earlier release of enzyme from DNA; therefore, the size of RNA product is smaller than the control.

Abbreviation: SSC, 15 mM NaCl–1.5 mM Na_3citrate.

MATERIALS AND METHODS

Rifampicin derivatives AF/05, AF/013, PR/19, and PR/14 were kindly provided by Dr. G. Lancini of Lepetit (for structures see ref. 3). α-Amanitin was purchased from Calbiochem.

DNA-dependent RNA polymerase II (or B) was isolated and purified from lymphocytes of patients with chronic lymphocytic leukemia by slight modification of the method of Roeder and Rutter (ref. 7 and unpublished results). Two forms of RNA polymerase were obtained from DEAE–Sephadex A-25 columns. RNA polymerase II was further purified by passage through phosphocellulose columns as described by Weaver *et al.* (8). Polymerase II, purified in this way, is free of ribonuclease, as judged by the rate of UMP incorporation, and RNA synthesis is completely DNA-dependent. Assay for RNA polymerase activity is described in the legend of each table or figure. Binding of polymerase to [H^3]DNA was determined by the Millipore filter method of Jones and Berg (9).

RNA was synthesized *in vitro* as described in the assay of RNA polymerase, except that the [^{3}H]UTP specific activity was 800 Ci/mol. At the end of the incubation period the reaction was stopped by heating at 80° for 2 min. The reaction mixture was then treated with 10 μg/ml of DNase I (RNase free) at room temperature (23°) for 30 min. Sodium dodecyl sulfate was added to a final concentration of 0.5%, and the mixture was extracted twice with an equal volume of phenol saturated with 0.1 × SSC [15 mM NaCl–1.5 mM Na_3citrate (pH 7.0)]. The RNA solution thus obtained was dialyzed against 2 liters of 0.01 × SSC for 4 hr at 4° and concentrated by flash evaporation.

Electrophoreses in 2.4% polyacrylamide gels were performed with Buchler polyanalyst electrophoresis equipment at 5 mA per tube for 1 hr; the gels were cut into 2-mm slices. Each gel was extracted twice by shaking with 1 ml of 2 × SSC –0.1% sodium dodecyl sulfate at 37° for 4 hr to isolate RNA. The RNA was precipitated by 5% Cl_3CCOOH. Cl_3CCOOH-precipitable material was collected on glass-membrane filters and counted in toluene-base solvent.

RESULTS

The effects of rifampicin and four of its derivatives on RNA polymerase II from lymphocytes of patients with chronic lymphocytic leukemia are shown in Table 1. PR/19, one of the rifampicin derivatives, can inhibit RNA synthesis only after being previously incubated with the enzyme at 37°, as in

the case shown for 2 min, before starting the polymerization reaction. If added immediately after the reaction is started, PR/19 exerted little effect on enzyme activity. This inhibition was not observed at concentrations of 0.02 and 0.10 M $(NH_4)_2SO_4$, as shown in experiment B of Table 1, but inhibition was observed at 0.14 M $(NH_4)_2SO_4$, as will be discussed later. These results can be explained in two ways: first, PR/19 blocks RNA synthesis through inhibition of steps before chain elongation and, second, the affinity of PR/19 for RNA polymerase from patients with chronic lymphocytic leukemia is weak; thus prior incubation of the drug and enzyme is necessary for its inhibition.

Rifamycins AF/013 and AF/05 can inhibit RNA synthesis either with or without prior incubation (Table 1). These data suggest that AF/013 and AF/05 may also inhibit steps other than binding or chain initiation. However, rifampicin and its derivative PR/14, even at concentrations as high as 200 μg/ml, exerted no effects on polymerase from chronic lymphocytic leukemics regardless of whether they were added before or after initiation of RNA synthesis. This agrees with results of other laboratories that rifampicin has no effect on the RNA polymerase isolated from higher organisms (1).

TABLE 1. *Effects of rifampicin and its derivatives on RNA synthesis*

Exp.	Antibiotics	(μg/ml)	$(NH_4)_2SO_4$ (M)	Activity (cpm) With preincubation	Activity (cpm) Without preincubation
A	Control	0	0.10	802	949
	Rifampicin	200	0.10	793	—
	PR/14	200	0.10	852	—
	PR/19	200	0.10	105	—
		100	0.10	—	871
	AF/05	200	0.10	54	—
		100	0.10	—	5
	AF/013	200	0.10	0	—
		100	0.10	—	13
B	Control	0	0.02	579	652
	PR/19	200	0.02	65	615
	Control	0	0.10	1289	1133
	PR/19	200	0.10	48	892

Enzyme preincubated with antibiotics at 37° for 2 min before reaction mixture (DNA, etc.) is added to start the reaction. Enzyme was added to the reaction mixtures at 0°. Reaction was started by pipeting the mixture to the tube containing antibiotics. RNA polymerase II from chronic lymphocytic leukemics was routinely assayed in 0.25 ml of 50 mM Tris·HCl (pH 7.9); 2 mM $MnCl_2$; 2 mM 2-mercaptoethanol; 0.125 mM each of GTP, ATP, and CTP; 0.05 mM [^{3}H]UTP (1.22×10^5 cpm/nmol); 20 μg of calf-thymus DNA; 5–10 μg of enzyme protein (7 units of enzyme activity); and $(NH_4)_2SO_4$ at the concentration indicated in the table. One unit of enzyme is defined as the amount of enzyme that can catalyze the incorporation of 1 pmol of UMP into Cl_3CCOOH-insoluble material in 10 min. Antibiotics were dissolved in dimethyl formamide to a concentration of 10 mg/ml as stock solution, and were added to the assay mixture at the desired concentration. Equivalent amounts of dimethyl formamide were also added to the control samples. Drug concentration of 200 μg/ml corresponds to about 0.23 mM. The reactions were incubated at 37° for 10 min, and 5 ml of cold 5% Cl_3CCOOH containing 0.01 M sodium pyrophosphate were added to stop the reaction. An equal amount of the appropriate drug was added to the control sample at this time in order to compensate for the quenching caused by the color of the drug. After standing at 0° for at least 15 min, Cl_3CCOOH-precipitable material were collected on glass membrane filters, washed with 5% Cl_3CCOOH–0.01 M sodium pyrophosphate, and counted in a liquid scintillation counter with a toluene-base counting solvent.

TABLE 2. *Effects of concentration of rifampicin derivatives on RNA synthesis*

Exp.	Antibiotics	μg/ml	cpm
A	Control	0	2180
	AF/05	20	2210
		60	578
		100	78
		140	116
		200	120
	AF/013	20	976
		40	965
		60	574
		100	136
		140	43
		200	21
B	Control	0	969
	PR/19	20	1340
		40	1400
		60	1060
		100	972
		140	622
		200	200

RNA polymerase II from chronic lymphocytic leukemics was preincubated with the desired amount of antibiotics at 37° for 2 min before the assay mixture was added to start the reaction. The reaction mixture and assay conditions were the same as described in the legend of Table 1. In experiment *A*, 16 units of enzyme were used; in experiment *B*, 8 units of enzyme were used per assay.

Studies of RNA synthesis as a function of drug concentration are shown in Table 2. The concentration of PR/19 required to inhibit RNA synthesis is at least 4-fold higher than that of AF/05 and AF/013, which may indicate that the binding of PR/19 to enzyme is weaker in comparison with the other two derivatives.

In order to investigate the modes of action exerted by PR/19, AF/05, and AF/013, the rates of [^{3}H]UMP incorporation into RNA in the presence of these rifampicin derivatives were monitored. As shown in Fig. 1, PR/19 had little, if any, effect on RNA synthesis if it was added after incubation of the complete reaction mixture at 37° for 5 min; however, AF/05 and AF/013 can inhibit incorporation up to 70% under the same conditions. Fig. 2 shows the same type of experiment at 0.02 M and 0.14 M $(NH_4)_2SO_4$; PR/19 once again had little effect on RNA synthesis, but at 0.14 M $(NH_4)_2SO_4$ more inhibition was observed. Since some variability was observed in the low-salt effects on PR/19, it may be unwise to draw strong conclusions at this point. At both salt concentrations, AF/05 remained the stronger inhibitor in comparison with PR/19. The effect of AF/05 is similar at all salt concentrations used.

It has been well documented that actinomycin D inhibits RNA polymerases by binding to DNA and blocking chain

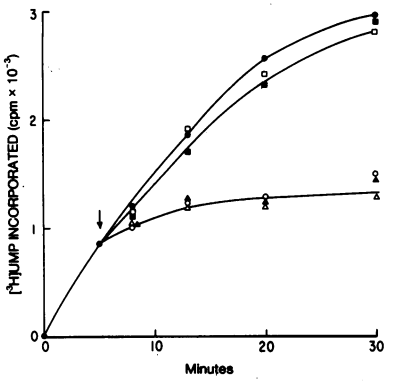

FIG. 1. Inhibition of RNA synthesis by rifampicin derivatives. Reaction was performed as described in Table 1, except that 1.1 ml of assay mixture was used. Final $(NH_4)_2SO_4$ concentration was 0.09 M. Rifampicin derivatives are added at 5 min as indicated by the *arrow;* 0.2 ml of each sample was taken out at intervals as indicated, and processed according to the method described in Table 1. ●——●, control (dimethyl formamide); ■——■, PR/19 (111 μg/ml); □——□, PR/19 (222 μg/ml); △——△, AF/05 (111 μg/ml); ▲——▲, AF/05 (222 μg/ml); ○——○, AF/013 (222 μg/ml).

elongation (10, 11). On the other hand, α-amanitin inhibits mammalian RNA polymerase II by binding to the enzyme (12, 13). We have studied both antibiotics in parallel with AF/05 and the results are shown in Fig. 3. Both actinomycin D and α-amanitin depressed [^{3}H]UMP incorporation rapidly, but [^{3}H]UMP incorporation continued for a few minutes in the presence of AF/05. Possible explanations for the mode of action of the drugs tested are as follows: PR/19 inhibits either initiation of RNA synthesis or a step before initiation; this can occur either in high ionic-strength medium or when enzyme is previously incubated with the drug. AF/013 and AF/05 appear to inhibit initiation or steps before initiation, and may also affect the elongation of RNA chains, even though they are not as effective as α-amanitin and actinomycin D.

To decide if AF/013 inhibits RNA chain elongation, we measured the molecular weight distribution of the RNA synthesized. Similarly, size analyses of the RNA was used to decide whether PR/19 affects both initiation and elongation

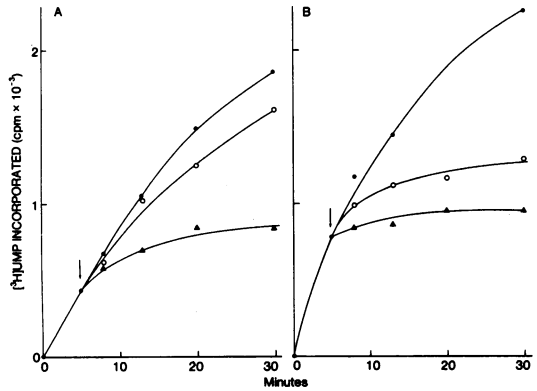

FIG. 2. Effectiveness of drug inhibition at low [*A*, 0.02 M] and high [*B*, 0.14 M $(NH_4)_2SO_4$] salt concentration. Reactions were performed as described in Fig. 1. Final drug concentration was 222 μg/ml for both PR/19 (○——○) and AF/05 (▲——▲). Other symbols are as in Fig. 1.

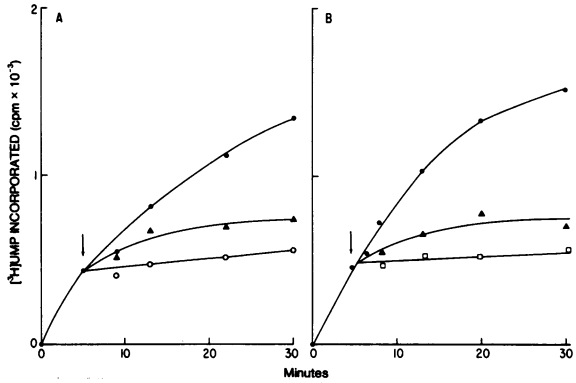

FIG. 3. Inhibitory effect of AF/05 (▲——▲), α-amanitin (□——□), and actinomycin D (○——○). Reactions were as in Fig. 1. Concentration of drugs was: AF/05, 111 μg/ml; actinomycin D, 50 μg/ml; and α-amanitin 1.11 μg/ml. ●——●, control; *arrow*, indicates time of drug addition.

at a concentration of 0.1 M $(NH_4)_2SO_4$. These results are summarized in Table 3, where the conditions of RNA synthesis are shown in the first column. In experiment A of Table 3, RNA polymerase II of chronic lymphocytic leukemics was added to the reaction mixture and incubated at 37° for 2 min before addition of drugs or drug solvent, dimethyl formamide. Reaction mixtures were then incubated an additional 13 min at 37°. In experiment B, a 100-fold excess of nonradioactive UTP was added at the time of the drug or solvent addition and subsequent incubation was as above, 13 min at 37°. The data in Table 3 show the average molecular weight as compared to the 2-min control, and the percentage increase

TABLE 3. *Effect of rifamycin derivatives AF/013 and PR/19 on RNA size*

Exp.		RNA synthesis condition*	Molecular weight (×10^{-5})	Molecular weight at 2 min (×10^{-5})	% increase
A.	1.	Incubation mixture + enzyme	0.98	0	—
	2.	Dimethyl formamide	1.35	0.37	100
	3.	AF/013	1.60	0.62	168
	4.	PR/19	1.32	0.34	92
B.	1.	Incubation mixture + enzyme	1.03	0	—
	2.	Dimethyl formamide + UTP	1.63	0.60	100
	3.	AF/013 + UTP	1.42	0.39	65
	4.	PR/19 + UTP	1.60	0.57	95

* Incubation mixture was as described in Table 1, except that the [^{3}H]UTP specific activity was 800 Ci/mol, and human-placenta DNA was used instead of calf-thymus DNA. Enzyme used in this experiment was 7 units, $(NH_4)_2SO_4$ concentration was 0.1 M, and drug concentration was 200 μg/ml. After incubation, RNA prepared *in vitro* was isolated and its size was determined in 2.4% polyacrylamide gel. All incubations were at 37°. Prior incubation of mixture + enzyme was for 2 min in all reactions. Incubation with rifamycin derivatives was for 13 min in all reactions.

in molecular weight when the RNA size in the solvent control is set at 100%. In experiment A, where reinitiation can occur, one expects the distribution of RNA sizes of the solvent control to be smaller than in those experiments where reinitiation is blocked (16). Therefore, the size of RNA synthesized in the presence of various drugs should follow the following trends:

Reinitiation inhibited > Solvent control > Elongation inhibited > 2-min control.

If initiation and elongation were both affected during RNA synthesis, we would expect the size of the RNA to be somewhere between the initiation-inhibited and elongation-inhibited size distributions. In experiment B, where unlabeled UTP is used in chase experiments, the size of RNA product should not be affected by reinitiation. Therefore, the order of size in this experiment can be arranged as follows:

Reinitiation inhibited = Solvent control > Elongation inhibited > 2-min control.

In Table 3, experiment A shows that the size of RNA follows the order: AF/013 > solvent control = PR/19 > 2-min control. This finding indicates that AF/013 inhibits chain initiation. The results with PR/19 in experiment A suggest that either (*a*) PR/19 has no inhibitory affect or (*b*) PR/19 is both an initiation and elongation inhibitor. Experiment B with solvent control = PR/19 > AF/013 > 2-min control indicates that AF/013 is also an elongation inhibitor and PR/19 prevents neither reinitiation nor elongation in 0.1 M $(NH_4)_2SO_4$.

These results led us to conclude that AF/013 inhibits RNA-chain reinitiation and consequently inhibits the increase of size of RNA by 35%. PR/19 has little, if any, effect on chain reinitiation and elongation once synthesis has begun. Since

TABLE 4. *Effect of PR/19 on binding of DNA to enzyme*

Condition		cpm retained on Millipore filter Exp. A	Exp. B
1. Enzyme preincubated with reaction mixture at 37° for 2 min before addition of drug	Control	1170	—
	PR/19 (200 μg/ml)	737	—
2. Enzyme preincubated with solvent or PR/19 at 37° for 2 min before to addition of reaction mixture	Control	913	1100
	Pr/19	150	196
	(200 μg/ml)	150	196
3. Enzyme preincubated with solvent or AF/013 at 37° for 2 min before addition of reaction mixture	Control	—	447
	AF/013 (200 μg/ml)	—	14

Assay mixtures are as described in Table 1 with the exception that 6 μg of lymphocyte [^{3}H]DNA (1000 cpm/μg) was substituted for calf-thymus DNA and unlabeled UTP was substituted for tritium-labeled UTP. Final $(NH_4)_2SO_4$ concentration was 0.09 M. Enzyme was preincubated with the reaction mixture or drug at 37° for 2 min, then the drug or reaction mixture was added and incubated at 37° for 2 additional min. The amount of DNA–enzyme complex was determined by filtration through Millipore filters as described by Jones and Berg (9). The blank containing the reaction mixture without enzyme showed 40 cpm retained on filters.

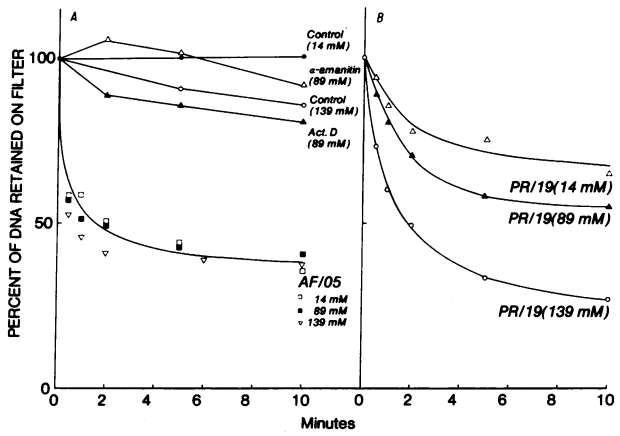

FIG. 4. Effect of drugs on the stability of the enzyme–DNA complex. Reaction was the same as indicated in Table 3, and $(NH_4)_2SO_4$ concentrations used are indicated in the figure. 50 Units of enzyme were incubated with 1.6-ml reaction mixture at 37° for 5 min. Drugs were then added and reaction was carried out for additional time period as indicated in the figure. DNA–enzyme complex was measured by the binding method of Jones and Berg (9). The amount of DNA retained on the Millipore filter at the time of addition of drug was set to be 100%. The concentrations of drugs used were: AF/05, 100 μg/ml; PR/19, 200 μg/ml; α-amanitin, 1 μg/ml; actinomycin D, 50 μg/ml. Controls contain equivalent amount of solvent. In the control sample, the zero-time value was 1828 cpm in 14 mM $(NH_4)_2SO_4$ and 1621 cpm in 139 mM $(NH_4)_2SO_4$.

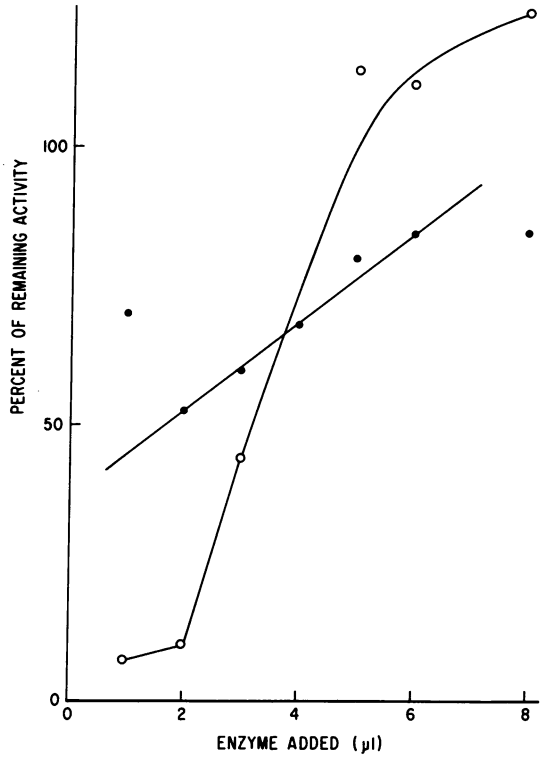

FIG. 5. Effect of enzyme concentration on drug inhibition. Reaction was performed as indicated in Table 1. Drug concentrations used in this experiment were PR/19 (●——●), 160 μg/ml; AF/05 (○——○), 40 μg/ml. Enzyme concentration used in this experiment was 2 μg/ml (1.4 units).

reinitiation is a multistep process including enzyme release, enzyme binding, and subsequent chain initiation, binding experiments of enzyme to DNA were performed in order to see whether AF/013 and PR/19 exert any affect on the binding step.

The binding of enzyme to [^{3}H]DNA was determined by the Millipore filter method of Jones and Berg (9). In this assay duplex [^{3}H]DNA bound to the enzyme is retained by Millipore filters, while free DNA passes through the filter. As shown in Table 4, enzyme–DNA complex formation can be reduced if the enzyme is previously incubated with PR/19 or AF/013. This finding indicates that rifamycin derivatives, PR/19 and AF/013, act differently from rifampicin; the latter shows no effect on binding (1, 5, 6).

Results of experiments designed to ask if PR/19 and AF/05 would prevent release of enzyme from DNA are shown in Fig. 4. Drugs were added after the reaction mixture had been incubated for 5 min. Samples were removed at the time intervals indicated, and the amounts of DNA–enzyme complex were measured by the Millipore technique (9). The results demonstrated that AF/05 does not prevent release of the enzyme, but apparently the enzyme is prevented from rebinding under various salt concentrations. Parallel experiments were also performed with actinomycin D and α-amanitin, where no appreciable dissociation of DNA–enzyme complex was observed. This result indicates that actinomycin D and α-amanitin stop RNA synthesis without releasing the enzyme from DNA. Similar experiments were done with PR/19 as shown in panel *B*. The action of PR/19 on formation of enzyme–DNA complex is greatly affected by salt concentration; the higher the salt concentration, the faster is the dissociation of the enzyme–DNA complex. This result is consistent with data shown in Figs. 1 and 2, which show that without prior incubation inhibition of RNA synthesis by PR/19 is manifest only at high salt concentrations. These results do not preclude the possibility that in high-salt solutions PR/19 increases the dissociation rate of the enzyme–DNA complex.

In order to determine whether the rifamycin derivatives act like rifampicin and inhibit RNA synthesis by binding to the RNA polymerase instead of to the DNA, we performed the experiments shown in Fig. 5. If rifamycin derivatives inhibit RNA synthesis by binding to DNA, one would expect that the percentage inhibition should not be affected by the amount of enzyme in the assay mixture. Inhibition was maximal at low concentrations of enzyme when the drug was in excess, but as the amount of enzyme was increased, the drug became limiting resulting in less inhibition. This observation suggests that the drugs inhibit RNA synthesis by interacting with RNA polymerase. From the data in Fig. 5 and Table 2, at high ratios of enzyme to drug, both AF/05 and PR/19 showed slight stimulatory effects on RNA synthesis.

DISCUSSION

The rifamycin derivatives studied here prevent the binding of RNA polymerase to DNA. Aside from inhibiting binding and initiation, AF/013 can prevent chain elongation resulting in a decrease in the size of RNA synthesized. While these studies were in progress, Meilhac *et al.* (3) reported the effect of rifamycin derivatives on calf-thymus RNA polymerase. They concluded that initiation, but not elongation, was inhibited by AF/05, and that both the primary binding of the enzymes to DNA and a temperature-dependent step were inhibited. If AF/05 and AF/013 act in the same way, then our results are at variance with the report of Meilhac *et al.*, whose conclusion that AF/05 does not effect elongation was based on the finding that AF/05 inhibits RNA synthesis in the same manner as polyethylene sulfate. Since the effect of polyethylene sulfate on chain elongation has not been clearly established (14), the interpretation that it effects chain elongation is questionable. The data presented in Table 3 clearly showed that AF/013 inhibits binding and elongation (size increased 65% as much as the dimethylformamide control). The strongly hydrophobic side chain of AF/05 (3) might suggest why it does not prevent the release of enzyme from DNA. However, PR/19 affects RNA synthesis by preventing binding of enzyme to DNA. This occurs either after prior incubation at low-salt concentration with enzyme or at high-salt [0.14 M $(NH_4)_2SO_4$] concentration, suggesting that the interaction between PR/19 and the enzyme is very weak, or it is a very slow process compared to the interaction between enzyme and DNA. Therefore, when the enzyme is released during transcription in low-salt medium it can then bind again to DNA before PR/19 has formed a stable complex with the enzyme. The higher the salt concentration, the faster is the dissociation rate of the enzyme–DNA complex (15), which makes RNA polymerase more sensitive to PR/19 in high-salt solution.

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