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STUDIES ON THE INHIBITION OF POLY(A) POLYMERASE FROM RAT LIVER AND HEPATOMA 3924A BY RIFAMYCIN SV DERIVATIVES

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Abstract—The inhibition of poly(A) polymerase activity from liver and hepatoma 3924A by several *O*-*n*-alkyl derivatives of rifamycin SV:3-formyloxime was studied. Only the *O*-*n*-pentyl (AF/012) and the *O*-*n*-octyl (AF/013) analogs were active at the maximum tested concentration of 1×10^{-4} M. Equivalent concentrations of liver and hepatoma enzymes were inhibited to the same degree by both compounds. The 50 per cent inhibitory concentration (IC_{50}) for AF/013 and AF/012 was 1.2×10^{-5} M and 7.5×10^{-5} M respectively. Poly(A) polymerase activity was more sensitive to AF/013 if the enzyme was preincubated with the drug before the initiation of the reaction with poly(A) than if the reaction was initiated by the addition of the enzyme. Addition of AF/013 after initiation of the assay led to a rapid inhibition of poly(A) synthesis. The mechanism of inhibition by AF/012 and AF/013 of poly(A) polymerase was of the non-competitive type with respect to ATP.

Analogs of rifamycin SV with substitution at the 3 position of the ansa ring of the molecule are powerful inhibitors of many eukaryotic polymerase enzymes [1–9]. The mechanisms of action of the semisynthetic rifamycin SV antibiotics have been studied with many primer–template-dependent enzymes including viral reverse transcriptase [3], and mammalian RNA [6, 7, 10] and DNA polymerases [4, 11]. In addition, the inhibitory actions of the drugs have been examined in the primer-dependent poly(A) ‡ polymerase reaction [8, 12, 13]. In some instances, differential inhibition of normal and leukemic DNA polymerases by certain rifamycin analogs has been reported [4, 11]. The majority of the studies with the primer–template-dependent enzymes indicates that the major effect of the rifamycin SV compounds can be attributed to the prevention of initiation of polymerization via formation of the enzyme–template–initiation complex [3, 6, 9], similarly to that seen with bacterial RNA polymerase [14]. This appears to be due to the binding of the drug to the enzyme and not to the primer–template [3, 7, 9], with the resultant effect of non-competitive inhibition [3, 9].

The mechanism of rifamycin inhibition of the poly(A) polymerase reaction, a primer-dependent reaction, has also been reported to result from a binding of the drug to the enzyme and not to the primer [8, 12, 13]. In contrast to the work with the other polymerase enzymes, the kinetics of inhibition were reported to be competitive with respect to the nucleotide substrate [8, 13].

In order to investigate the mechanism of polyadenylylation with normal and neoplastic enzymes, and to verify that the primer-dependent poly(A) polymerase reaction was indeed different from the primer–template-dependent polymerase reactions, poly(A) polymerase was highly purified from rat liver and hepatoma 3924A. The extent and mode of inhibition of both enzymes were investigated with several *O*-*n*-alkyl derivatives of rifamycin SV:3-formyloxime. The results of this report are in agreement with the major aspects of the previous reports dealing with RNA polymerase, DNA polymerase, and reverse transcriptase but diverge in several aspects from earlier studies with poly(A) polymerase [8, 12, 13, 15, 16].

MATERIALS AND METHODS

Chemicals. [2,8-^{3}H]ATP (40 Ci/m-mole), [α-^{32}P]ATP (20 Ci/m-mole), and Omnifluor were obtained from New England Nuclear, Boston, MA. Phosphocellulose (Whatman P-1) and glass fiber filter discs (Reeve Angel, 934AH) were purchased from Whatman, Inc., Clifton, NJ. ATP, poly(A), and poly(U) were obtained from the Sigma Chemical Co., St. Louis, MO. Oligo(rA)$_{25}$, and oligo(rA)$_{45}$ were purchased from the Miles Laboratories, Elkhart, IN.

Animals. Male Sprague–Dawley rats were purchased from ARS/Sprague–Dawley, Madison, WI, and were the source of normal liver. ACI/N rats (Laboratory Supply Co., Indianapolis, IN) bearing hepatoma 3924A transplanted intramuscularly were obtained

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‡ Abbreviations used are: poly(A), polyriboadenylic acid; poly(U), polyribouridylic acid; HnRNA, heterogeneous nuclear RNA; PGEM buffer, 40 mM potassium phosphate (pH 6.8), 20% (v/v) glycerol, 0.1 mM EDTA, and 2 mM 2-mercaptoethanol; TGMEM buffer, 50 mM Tris–HCl (pH 7.9), 25% (v/v) glycerol, 5 mM $MgCl_2$, 0.1 mM EDTA, and 2 mM 2-mercaptoethanol; DMF, dimethylformamide; and IC_{50}, 50 per cent inhibitory concentration.

from Dr. H. P. Morris, Washington, DC. All animals were maintained two per cage, containing aspen wood shavings as bedding with alternating 12-hr periods of darkness and light. All animals were allowed access to food and water as needed.

All rifamycin antibiotics were stored in a vacuum dessicator at 4° and kept protected from light. Solutions of rifamycin analogs in DMF were made fresh before use since it was found that the drugs in solution were subject to photodestruction within 48 hr at either 4° or 20°. The final concentration of DMF in the assay was 1.7% (w/v) and did not affect poly(A) polymerase activity.

Isolation of liver and hepatoma nuclei. Nuclei were prepared by rapid perfusion of the excised liver with ice-cold 0.25 M sucrose–5 mM $MgCl_2$. A 12% (w/v) homogenate of the liver was prepared in 2.4 M sucrose–10 mM $MgCl_2$ using six strokes of a motor-driven Teflon-glass homogenizer. Hepatoma nuclei, due to their different densities, were isolated in a similar manner except that a 14% (w/v) homogenate was prepared. All subsequent steps were the same for both tissues. The liver or hepatoma homogenate was centrifuged at 40,000 *g* for 60 min. The pellet was washed with ice-cold 0.34 M sucrose–5 mM $MgCl_2$ and resuspended in 5 ml of the same solution. The nuclei were layered over 5 ml of 0.88 M sucrose–5 mM $MgCl_2$ and centrifuged at 2,000 *g* for 20 min. The pellet containing purified nuclei has been previously shown by electron microscopy to be free of cytoplasmic tags [17].

Solubilization and chromatography of poly(A) polymerase. Poly(A) polymerase was solubilized by suspension of the nuclei in TGMEM buffer (1 ml/g wet wt of liver), addition of saturated $(NH_4)_2SO_4$ (adjusted to pH 7.9 with NH_4OH) to 0.3 M, and sonication with a microtip probe at setting 3 (Heat Systems-Ultrasonics, Plainview, NY) for six 10-sec intervals at 4°. The disrupted nuclei were dialyzed against 60 volumes of PGEM buffer for 3 hr and centrifuged at 100,000 *g* for 30 min. The supernatant fluid was adsorbed to a phosphocellulose column (1.5 × 30) and poly(A) polymerase activity was eluted with a linear gradient of 0 to 0.5 M KCl in PGEM buffer. Peak fractions containing poly(A) polymerase activity were pooled and dialyzed against 50 volumes of PGEM buffer for 2 hr. The dialyzed material was adsorbed to a poly(A)-Sepharose column (0.9 × 10) equilibrated with PGEM buffer. The column was washed with 2 volumes of PGEM buffer and the enzyme activity was eluted with 2 volumes of 1.0 M KCl–PGEM buffer. Poly(A) polymerase was stored at −80° in 1.0 M KCl–PGEM buffer that had been adjusted to 50% (v/v) glycerol. The enzyme activity was stable under these conditions for 4 months.

Poly(A) polymerase assay. Poly(A) polymerase was routinely assayed for 15 min at 37°. The assay mixture in a final volume of 0.3 ml contained: 70 mM Tris–HCl (pH 7.9), 0.4 mM $MnCl_2$, 0.1 mM ATP, 0.5 μCi [^{3}H]ATP (33.3 mCi/m-mole), 10 μg poly(A) and 20–50 μl enzyme. In studies with the rifamycin SV analogs, the assay was initiated by the addition of 1 μg liver or hepatoma enzyme, unless noted otherwise. The reaction was terminated by the addition of 3 ml of ice-cold 5% trichloroacetic acid–10 mM $Na_4P_2O_7$. The precipitates were collected on two glass fiber filters and washed successively first with 10 ml of 5% trichloroacetic acid–10 mM $Na_4P_2O_7$ and then with 10 ml of 95% ethanol, and dried. Radioactivity was determined in a Beckman LS-335 liquid scintillation system after the addition of 5 ml of Omnifluor–Triton X-100–toluene (5 g:333 ml:666 ml) scintillation fluid. One unit of activity is defined as that amount of enzyme which incorporates 1 pmole AMP/min into acid-insoluble material.

Preparation of poly(A)-Sepharose. CNBr-Sepharose 4B was prepared as described [18]. A 10-ml solution containing 2 mg/ml of poly(A) in 0.2 M MES buffer (pH 6.0) was added to 10 g CNBr-Sepharose and stirred overnight at room temperature. After filtration, poly(A)-Sepharose was washed with cold water, and unreacted CNBr-Sepharose was inactivated in 1 M glycine. After further washing with water, Poly(A)-Sepharose was stored at 4° in PGEM buffer. The coupling of poly(A) was greater than 85 per cent and was stable for 4 months.

Preparation of poly(U)-Sepharose. Poly(U) was coupled to CNBr-Sepharose according to the Lindberg and Persson [19].

Preparation of poly(A)-RNA primers. HnRNA was extracted from liver nuclei at room temperature using 1 volume of 0.3% sodium dodecyl sulfate–Tris–HCl (pH 9.0) buffer and 1 volume of a mixture of phenol–cresol–water (7:2:1, v/v) containing 0.1% 8-hydroxyquinoline. Polysomal RNA was extracted from Mg^{2+}-precipitated polysomes [20] as described for HnRNA except that 0.5 volume of phenol mixture was initially mixed with the sodium dodecyl sulfate solution of RNA followed by 0.5 volume of chloroform. The RNA was precipitated with 2 volumes of 2% (w/v) potassium acetate in 95% ethanol overnight at −20°, and centrifuged at 25,000 *g* for 20 min. The RNA was further washed with ice-cold 95% ethanol, centrifuged at 25,000 *g* for 10 min, and drained dry. Poly(A)-and non-poly(A)-RNA were separated by poly(U)-Sepharose affinity chromatography as described [21]. Poly(A)- and non-poly(A)-RNA were precipitated and washed with ethanol as described above. RNA fractions were dissolved in water and frozen at −10° until used.

Nearest-neighbor analysis. Liver and hepatoma poly(A) polymerase were incubated without exogenous primer for 45 min in the presence of 3 μCi [α-^{32}P]ATP (100 mCi/m-mole). The reaction was terminated by the addition of 5% trichloroacetic acid–10 mM $Na_4P_2O_7$, and washed as described above. The precipitate was dissolved in 0.3 M KOH and incubated overnight at 37°. Nucleotides were separated by chromatography on standardized columns of Dowex-H^+ and Dowex-formate, as described by Katz and Comb [22]. The samples were lyophilized and radioactivity was determined after the addition of 5 ml of Omnifluor–Triton X-100–toluene scintillation fluid. The average chain length was computed from the ratio of [^{32}P]AMP to the sum of ^{32}P in all other nucleotides [23].

Protein determination. Protein concentrations were determined by the procedure of Lowry *et al.* [24].

Kinetic analysis. All data were plotted according to the Hanes modification of the Michaelis–Menten equation [25]. In this analysis, the straight line equation is $S/V = (1/V_m)(S) + K_m/V_m$.

Table 1. Purification of liver and hepatoma poly(A) polymerase*

Fraction	Total activity (10^3 units)	Total protein (mg)	Specific activity (10^3 units/mg protein)	Recovery of protein (%)	Purification (-fold)
Liver					
Nuclear extract	44.8	9.8	4.6	100	1
Phosphocellulose	5.9	0.3	19.7	3	4
Poly(A)-Sepharose	3.1	0.03	103.3	0.3	22
Hepatoma					
Nuclear extract	15.2	14.5	1.0	100	1
Phosphocellulose	2.9	0.8	3.6	5	4
Poly(A)-Sepharose	1.5	0.1	15.0	0.8	15

*Poly(A) polymerase was purified from nuclei obtained from approximately 25 g liver or 40 g hepatoma 3924A, as described in Materials and Methods. Preparations obtained from liver are the average of four procedures (range = 52,000 to 260,000 units/mg of protein; 11- to 57-fold purification), and preparations from hepatoma 3924A are the average of five procedures (range = 9,000 to 18,000 units/mg of protein; 9- to 18-fold purification).

RESULTS

Purification of poly(A) polymerase activity from liver and hepatoma. Poly(A) polymerase activity from isolated nuclei was eluted from phosphocellulose as a single peak with a linear KCl salt gradient in a pH 6.8 buffer. The pooled fractions were dialyzed against a low salt buffer and applied to a poly(A)-Sepharose affinity column. The enzyme activity eluted as a single sharp peak when the column was washed with 1.0 M KCl buffer. Table 1 shows the average purification using this rapid two-step procedure. Purification of 22- and 15-fold over the nuclear extract was achieved for the liver and hepatoma enzymes, respectively. The specific activity of hepatic poly(A) polymerase was 5-fold higher than hepatoma poly(A) polymerase in the nuclear extract, and remained 7-fold greater after poly(A)-Sepharose chromatography. No chromatographic differences between the liver and hepatoma enzymes were noted.

Divalent metal requirement. Liver and hepatoma poly(A) polymerase activity exhibited an absolute requirement for Mn^{2+}, while Mg^{2+} was unable to substitute for Mn^{2+} at concentrations of $0.2–3 \times 10^{-3}$ M (Table 2). The apparent K_m for either enzyme was approximately 9×10^{-5} M, and maximal reaction rates were obtained with 4×10^{-4} M $MnCl_2$.

Nucleotide requirement. ATP was preferentially utilized by poly(A) polymerase, whereas either TTP or ADP with poly(A) as primer or UTP with poly(U) as primer did not result in measurable activity (Table 2). The K_m for ATP was 5×10^{-5} M and 6×10^{-5} M for liver and hepatoma enzymes, respectively. The optimal ATP concentration was 1×10^{-4} M, but concentrations higher than four times the K_m resulted in inhibition.

Primer requirements. For routine determination of poly(A) polymerase activity, poly(A) greater than 100,000 daltons was used as primer. The optimal concentration of poly(A) ranged from 33 to 66 μg/ml.

Table 2. Substrate and ion dependence of poly(A) polymerase*

Conditions	Poly(A) polymerase	
	Liver (% Incorporation)	Hepatoma (% Incorporation)
Complete: poly(A), Mn^{2+}, [^{3}H]ATP	100	100
−[^{3}H]ATP	<1	<1
−[^{3}H]ATP, + [^{3}H]TTP	<1	<1
−poly(A), −[^{3}H]ATP; + poly(U), + [^{3}H]UTP	<1	ND†
−[^{3}H]ATP, + [^{3}H]ADP	<1	ND
−poly(A)	10	1
−Mn^{2+}	<1	<1
−Mn^{2+}, + 0.2–3.0 mM Mg^{2+}	<1	<1
−poly(A), + nuclear non-poly(A)-RNA	8	10
−poly(A), + nuclear poly(A)-RNA	42	30
−poly(A), + polysomal non-poly(A)-RNA	3	3
−poly(A), + polysomal poly(A)-RNA	100	50

*Liver and hepatoma poly(A) polymerases were assayed for 15 min under optimal conditions, as described in Materials and Methods. The concentration of primer was 1 A_{260} unit/assay.

†Not determined.

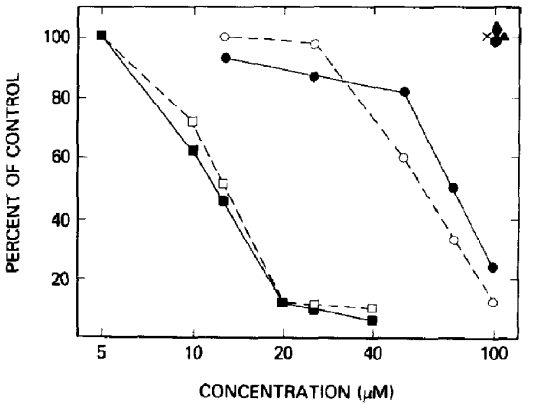

Fig. 1. Dose–response curves by derivatives of rifamycin SV upon poly(A) polymerase activity. AF/EO (▲), AF/08 (●), AF/09 (◆), AF/010 (×), AF/012 (●, ○), or AF/013 (■, □) were incubated for 15 min with 1 μg liver or hepatoma poly(A) polymerase under optimal conditions. All points are the average of duplicate determinations. Solid symbols represent liver enzyme; open symbols represent hepatoma enzyme. Control values (units) for the liver and hepatoma enzymes were 100 and 15 respectively.

Concentrations above the optimum resulted in lower enzyme activity and may be attributed to either non-specific binding of the enzyme to non-priming sites on poly(A) or to the sequestering of Mn^{2+} in the assay mixture.

The poly(A) polymerase enzymes from both liver and hepatoma were also tested for their ability to utilize endogenous RNA molecules as primers (Table 2). Nuclear and polysomal RNA from rat liver were separated into poly(A)- and non-poly(A)-RNA by poly(U)-Sepharose affinity chromatography. Non-poly(A)-RNA of nuclear or polysomal origin was not very effective as a primer for either enzyme; however, nuclear poly(A)-RNA and polysomal poly(A)-RNA were 30–40 and 50–100 per cent, respectively, as effective as synthetic poly(A).

The chain length of the reaction product of the poly(A) polymerase reaction was estimated by nearest-neighbor analysis using [α-^{32}P]ATP and endogenous primer. In this manner, it was estimated that the chain length of the product formed by the liver and the hepatoma enzymes was 25 and 8 nucleotides, respectively, after a 45-min incubation.

Dose–response of rifamycin analogs on poly(A) polymerase. A number of derivatives of rifamycin SV that have been shown to inhibit DNA-dependent polymerases were studied with the partially purified poly(A) polymerases from liver and hepatoma. Figure 1 shows the dose–response curves for the various rifamycin SV derivatives tested when the reaction was initiated by the addition of 1 μg liver or hepatoma poly(A) polymerase. No significant differences in sensitivity between the two enzymes to the rifamycin analogs were noted. Only the *O*-*n*-octyl (AF/013) and the *O*-*n*-pentyl (AF/012) derivatives were active against these enzymes at concentrations up to 100 μM. Rifamycin SV analogs with ethyl, *n*-propyl, isopropyl, or *n*-butyl substitutions were not active against these enzymes at the concentrations tested. The IC_{50} for AF/013 and AF/012, respectively, was 1.2×10^{-5} M and 7.5×10^{-5} M with either enzyme. The IC_{50} for AF/013 is in general agreement with those values reported by others for poly(A) polymerase and other polymerase enzymes. No previous estimation of the IC_{50} for AF/012 has been made for poly(A) polymerase.

The relationship between substrate concentration and enzyme activity in the presence of inhibitor is shown in Fig. 2. Under conditions were the assay was initiated by the addition of poly(A) to the enzyme–ATP–drug complex formed at 4°, both liver (Fig. 2A) and hepatoma (Fig. 2B) enzymes were completely inhibited by concentrations of AF/013 that only partially inhibited the enzyme activity when the reaction

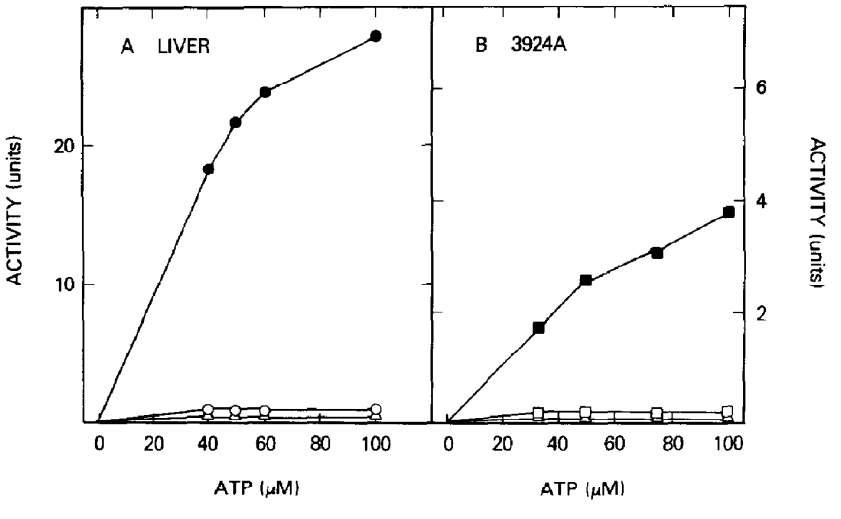

Fig. 2. Effects of AF/013 on poly(A) polymerase activity when added to the enzyme before initiation of the reaction. One μg of either liver or hepatoma poly(A) polymerase enzyme was added to all other components of the assay including the indicated concentrations of ATP and AF/013 without primer at 0°. The reaction was initiated by the addition of 10 μg poly(A). The activity was assayed for 15 min. Panel A: liver poly(A) polymerase; DMF (●), 1.5×10^{-5} M AF/013 (○), and 1.75×10^{-5} M AF/013 (△). Panel B: hepatoma poly(A) polymerase; DMF (■), 1.5×10^{-5} M AF/013 (□), and 1.75×10^{-5} M AF/013 (△).

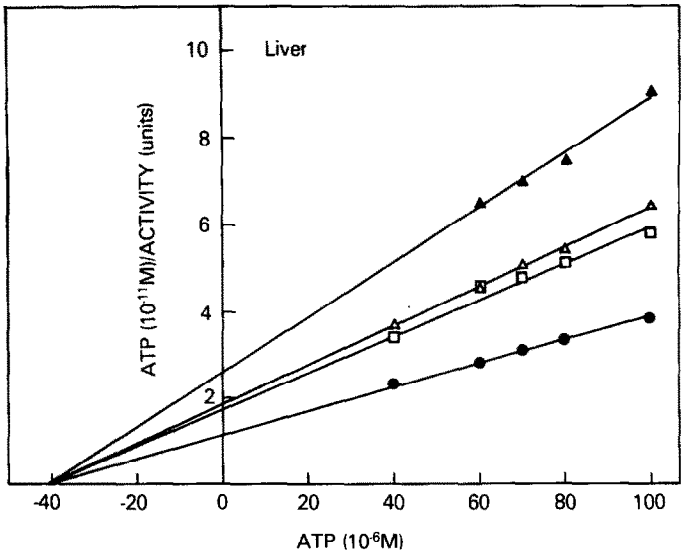

Fig. 3. S/V vs S plot of the inhibition of liver poly(A) polymerase activity by AF/012 and AF/013. Liver enzyme was incubated for 15 min at the indicated ATP concentrations with DMF (●), 5×10^{-5} M AF/012 (□), 1.5×10^{-5} M AF/013 (△) or 1.75×10^{-5} M AF/013 (▲). All points are the average of duplicate determinations which did not differ by more than 5 per cent.

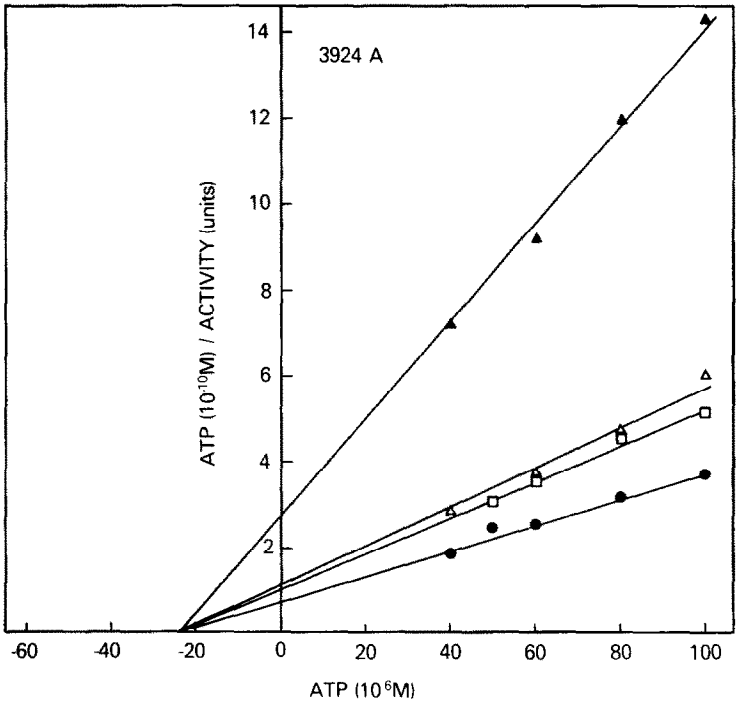

Fig. 4. S/V vs S plot of the inhibition of hepatoma poly(A) polymerase activity by AF/012 and AF/013. Hepatoma enzyme was incubated for 15 min at the indicated ATP concentrations with DMF (●), 5×10^{-5} M AF/012 (□), 1.5×10^{-5} M AF/013 (△), or 1.75×10^{-5} M AF/013 (▲). All points are the average of duplicate determinations which did not differ by more than 5 per cent.

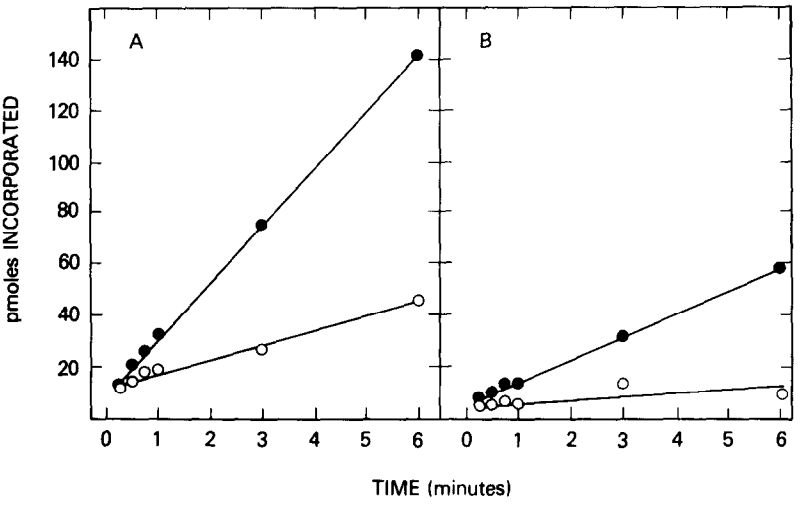

Fig. 5. Inhibition of elongation of the poly(A) chain by addition of AF/013 to the reaction. Liver poly(A) polymerase was incubated for 5 min with all assay components except [^{3}H]ATP. After 5 min, 5 μCi [^{3}H]ATP and either DMF (●) or 1×10^{-5} M AF/013 (○) were added to the assay and 300-μl aliquots were withdrawn at the indicated times to assay poly(A) polymerase activity. Panel A: the assay contained 1×10^{-4} M ATP. Panel B: the assay contained 3×10^{-4} M ATP.

was initiated by the addition of enzyme (Fig. 1). When the amount of protein in the assay mixture was varied, the concentration of AF/013 necessary to effect the same degree of inhibition was increased; however, an increase in the primer concentration did not alter the degree of inhibition with a given concentration of AF/013 (results not shown). These results suggest that AF/013 interacts primarily with the enzyme and not with the primer, and are in agreement with other studies [8, 26].

Kinetic analysis of the mode of inhibition exerted by AF/012 and AF/013. To evaluate the mode of inhibition of AF/012 and AF/013, concentrations of inhibitors were under assay conditions employing optimal ATP concentrations and in which the reaction was initiated by the addition of the enzyme. The results of these experiments are shown in Figs. 3 and 4. Liver poly(A) polymerase was inhibited in a non-competitive manner by AF/012 and AF/013 as indicated by the intersection of the lines on the abscissa (Fig. 3). When similar experiments were conducted with the hepatoma enzyme, non-competitive inhibition by AF/012 and AF/013 was obtained in all instances (Fig. 4).

Additional evidence indicating non-competitive inhibition by AF/013 is shown in Fig. 5. In these experiments, the reaction was initiated with 1×10^{-4} M ATP in the absence of [^{3}H]ATP and AF/013. Five min after the elongation of the polynucleotide chain had begun, [^{3}H]ATP and either DMF or 1×10^{-5} M AF/013 were added to the assay mixture and the amount of radio activity incorporated was determined at various times. As shown in Fig. 5A, the enzyme reaction was inhibited markedly within minutes after the introduction of AF/013. In Fig. 5B, the assay was carried out in a similar manner except that the reaction was initiated by the addition of 3×10^{-4} M ATP. The presence of higher levels of ATP did not reverse the inhibition of AF/013. In fact, the activity was lowered as a result of substrate inhibition.

DISCUSSION

Poly(A) polymerases from isolated nuclei of rat liver and from a poorly differentiated transplantable hepatoma were partially purified by a rapid two-step procedure. After chromatography on phosphocellulose and poly(A)-Sepharose, purifications of approximately 22- and 15-fold could routinely be effected for the liver and hepatoma enzymes respectively. The average specific activities of the liver and hepatoma enzymes were 3.5- and 1.2-fold greater, respectively, than those previously reported [27]. The wide range of specific activities reflected our earlier attempts at purification, and the activity for the liver enzyme is close to the specific activity of pure hepatic poly(A) polymerase recently reported [28]. Therefore, our enzyme preparations, while not 100 per cent pure, were highly purified. Moreover, the enzymes isolated from these tissues exhibited all of the characteristics that have been established for poly(A) polymerase, viz. utilization of ATP as the sole nucleotide substrate, preference for Mn^{2+}, and recognition of only oligo(A) region for the initiation of the reaction.

The ability of derivatives of rifamycin SV to inhibit the polymerase activity of normal and neoplastic tissue appears to be related to the degree of hydrophobic substitution of the ansa ring [1, 4, 15, 29]. The results of our experiments with normal and hepatoma poly(A) polymerase using rifamycin SV analogs with various aliphatic substituents would seem to support this theory. Only aliphatic substitutions of 5 carbons or longer were active against poly(A) polymerase at concentrations up to 1×10^{-4} M. The IC_{50} values for the *O-n*-pentyl (AF/012) and *O-n*-octyl (AF/013) derivatives indicate that the addition of a 3-carbon chain increases the effectiveness of the inhibitor 5-fold. On the other hand, decreasing the side chain by 1 carbon from *n*-pentyl to *n*-butyl results in a loss of inhibitory activity over the concentrations tested. This information may

prove to be of great value in the understanding of the active site(s) of the poly(A) polymerase enzyme once the binding site of the rifamycin molecule to the enzyme has been established. It would seem that the spatial position of the substituted group is more important than the size of the substitution, since larger groups have been previously shown not to inhibit various polymerases [15, 29].

The data indicate that the inhibition of the poly(A) polymerase reaction by the two active derivatives of rifamycin SV was non-competitive with respect to ATP. This observation is in agreement with the non-competitive kinetics reported for RNA polymerase [6, 7], reverse transcriptase [3], and DNA polymerase [9]. However, these results are in contrast to studies of the inhibition of poly(A) polymerase by AF/013 where competitive inhibition with respect to ATP was observed [8, 12, 13]. We have not found competitive kinetics at any concentration of rifamycin tested. In addition, higher concentrations of ATP did not reverse the inhibition of AF/013. It is possible that the greater levels of protein, substrate, and primer necessary to achieve optimal activity in the previous studies [8, 12, 13] altered the kinetics of the reaction. However, the kinetics of inhibition of AF/013 with RNA polymerase was also found to be competitive with respect to nucleotide concentration [8], a result also contrary to other reports [6, 7].

The present results support the finding that the elongation of the oligo(A) chain is rapidly inhibited by AF/013 [8, 12, 13], but differs from studies reporting that elongation is protected in a manner similar to that with DNA and RNA polymerases [15]. The limited protection of the enzyme by the presence of primer compared to that seen with DNA and RNA polymerases [6, 15], in conjunction with the rapid cessation of the ongoing enzyme reaction by the addition of AF/013, indicates that the elongation reaction of poly(A) polymerase is as sensitive as the initiation step in the DNA and RNA polymerase reactions. It has been suggested that the initiation of the poly(A) polymerase reaction is analogous to the RNA polymerase reaction [16], but the present results would not support this theory. Perhaps the formation of an enzyme–primer–template complex in the DNA and RNA polymerase reactions alters the conformation of the enzyme molecule in a manner not found in the primer–enzyme complex of the poly(A) polymerase reaction, thereby making it resistant to the binding of the rifamycin analog. Also, the poly(A) polymerase enzyme may release the primer after the addition of each AMP residue, thereby rendering it susceptible to attack by the rifamycin inhibitor at multiple points during the reaction.

This is the first report comparing the effects of various rifamycin SV derivatives on poly(A) polymerase from normal and neoplastic tissue. We could detect neither a difference in the sensitivity between the enzymes, nor the manner in which they were inhibited by the drugs. This agrees with other studies [4, 11] where AF/013 did not differentially inhibit DNA polymerases from normal or leukemic lymphoblasts. Derivatives that were reported to differentially affect normal and neoplastic DNA polymerase were not tested in this study.

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REFERENCES

1. C. Gurgo, R. Ray and M. Green, *J. natn. Cancer Inst.* **49**, 61 (1972).
2. G. F. Gerard, C. Gurgo, D. P. Grandgenett and M. Green, *Biochem. biophys. Res. Commun.* **53**, 194 (1973).
3. A. N. Wu and R. C. Gallo, *Biochim. biophys. Acta* **340**, 419 (1974).
4. S. S. Yang, F. M. Herrera, R. G. Smith, M. S. Reitz, G. Lancini, R. C. Ting and R. C. Gallo, *J. natn. Cancer Inst.* **49**, 7 (1972).
5. P. P. Juhaz, J. J. Benecke and K. H. Seifart, *Fedn Eur. Biochem. Soc. Lett.* **27**, 30 (1972).
6. M. Meilhac, Z. Tysper and P. Chambon, *Eur. J. Biochem.* **28**, 291 (1972).
7. M. H. Tsai and G. F. Saunders, *Proc. natn. Acad. Sci. U.S.A.* **70**, 2072 (1973).
8. K. M. Rose, P. A. Ruch and S. T. Jacobs, *Biochemistry* **14**, 3598 (1975).
9. M. Green, C. Gurgo, G. F. Gerard, D. Grandgenett and K. Shimada, in *Symposium on Fundamental Cancer Research*, p. 258. Williams & Wilkins, Baltimore (1974).
10. M. Meilhac and P. Chambon, *Eur. J. Biochem.* **35**, 454 (1973).
11. R. C. Gallo, J. W. Abrell, M. S. Roberts, S. S. Yang and R. G. Smith, *J. natn. Cancer Inst.* **48**, 1185 (1972).
12. S. T. Jacob and K. M. Rose, *Nucleic Acid Res.* **1**, 1549 (1974).
13. S. T. Jacob, K. M. Rose and J. M. Glazer, *Archs Biochem. Biophys.* **164**, 771 (1974).
14. W. Wehrli and M. Staehelin, *Bact. Rev.* **35**, 290 (1971).
15. V. S. Sethi and P. Okano, *Biochim. biophys. Acta* **454**, 230 (1976).
16. V. S. Sethi, *Fedn Eur. Biochem. Soc. Lett.* **59**, 3 (1975).
17. R. I. Glazer and M. F. LaVia, *Cancer Res.* **35**, 2511 (1975).
18. A. F. Wagner, R. L. Bugianesi and T. Y. Shen, *Biochem. biophys. Res. Commun.* **45**, 184 (1971).
19. U. Lindberg and T. Persson, *Eur. J. Biochem.* **31**, 246 (1972).
20. R. D. Palmiter, *Biochemistry* **13**, 3606 (1974).
21. J. J. Eiden and J. L. Nichols, *Biochemistry* **21**, 3952 (1973).
22. S. Katz and D. G. Comb, *J. biol. Chem.* **238**, 3065 (1963).
23. M. A. Winters and M. Edmonds, *J. biol. Chem.* **248**, 4763 (1973).
24. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
25. C. S. Hanes, *Biochem. J.* **26**, 1406 (1932).
26. S. Riva, A. Fietta and G. Silvestri, *Biochem. biophys. Res. Commun.* **49**, 1263 (1972).
27. K. Rose and S. T. Jacob, *Eur. J. Biochem.* **67**, 11 (1976).
28. D. Antoniades and O. Antonoglou, *Biochim. biophys. Acta* **519**, 447 (1978).
29. C. Gurgo, *Pharmac. Ther.* (A) **2**, 139 (1977).