Volume 3 no.2 February 1976 Nucleic Acids Research

Inhibition of RNA-directed DNA polymerase by aurintricarboxylic acid.

Jean F. Givens and Kenneth F. Manly

Department of Medical Viral Oncology, Roswell Park Memorial Institute, Buffalo, NY 14263, USA.

Received 11 December 1975

ABSTRACT

Commercial-grade aurintricarboxylic acid (ATA) inhibits poly(A), poly(C) and viral RNA-directed DNA synthesis by detergent-disrupted virions of Moloney murine leukemia virus. Paper chromatography of crude ATA yields two active components, which appear to behave identically, and at least two inactive components. The concentration of ATA needed to inhibit polymerase activity is proportional to the concentration of viral protein. The inhibition is neither attributable to contaminating heavy metal ions in the ATA preparation nor to chelation by ATA of Mn^{2+} or Zn^{2+}, the necessary co-factors. Inhibition of the polymerase reaction by ATA greatly increases the K_m for the primer [oligo(T)/oligo(dG)], while it only slightly lowers the V_{max} and does not affect the K_m's for the template [poly(A)/poly(C)] or the substrate (TTP/dGTP). Thus, ATA seems to reduce specifically the affinity of the polymerase for the DNA primer molecule.

INTRODUCTION

The triphenylmethane dye aurintricarboxylic acid (ATA)[1] has been shown to inhibit protein synthesis *in vitro*[2-7]. This is accomplished at low concentrations by preventing the attachment of mRNA to ribosomes and at higher concentrations by inhibiting the elongation step in protein synthesis. ATA also inhibits Qβ replicase and the RNA polymerases associated with *E. coli*, T7, and vesicular stomatitis virus by combining with the template binding sites on these enzymes, thus preventing initiation[8-10]. It has also been reported that ATA inhibits the RNA-directed DNA polymerase associated with avian myeloblastosis virus[11] and Rauscher murine leukemia virus[9]. We show in this report that a major effect of ATA is interference with the ability of RNA-directed DNA polymerase to bind a DNA primer molecule.

MATERIALS AND METHODS

Virus: Moloney murine leukemia virus, grown in BALB/c mice, was obtained from R.J. Eckner in the form of a cell-free homogenate of a leukemic spleen and was grown in JLS-V9 cells as previously described[12].

Polynucleotides: Poly(A) and poly(C), with a length greater than 250 nucleotides, as measured by gel filtration, was obtained from Miles Laboratories, Inc. Oligo(T) and oligo(dG), obtained from Collaborative Research, Inc. were mixtures of oligomers with lengths from 15 to 18 nucleotides. Concentrations of poly- and oligonucleotides are expressed as concentrations of monomers.

Purification of DNA polymerase: Virus-associated DNA polymerase was prepared from purified Moloney leukemia virus by the method of Faras et al[13]. Briefly, this method includes column chromatography of DEAE-cellulose, phosphocellulose, and gel filtration through Sephadex G-100.

Purification of the Inhibitor: "Aluminon" grade ATA, obtained from Fisher Scientific Co., was purified by two dimensional ascending chromatography on Whatman 3MM paper. The chromatogram was first developed in the aqueous phase, dried and then developed in the butanol phase of a 1-butanol, acetic acid, and water mixture (20:3:25). Four visible components were evident: a large yellow, a purple and two separate red spots. There is also at least one non-inhibiting UV-absorbing component[8] (and personal observation) not detected on this chromatogram but detectable on chromatograms with a fluorescent indicator. The components were eluted from the paper with a triethylamine carbonate-methanol solution and then lyophilized. The inhibitor(s) co-migrated with the two red components (fractions I and II) which have approximately equal inhibitory activity of the poly(A)-directed polymerase reaction.

Chelex-ATA refers to a preparation of "Aluminon" grade ATA which was passed through a column of Bio-Rad Chelex-100 to remove contaminating heavy metal ions.

Polymerase Assays: Unless otherwise noted, polymerase reaction mixes had a final volume of 50 µl, with the following components: 50 mM Tris-HCl buffer, pH 7.9, 20 µM dithiothreitol, 60 mM NaCl, 0.05% Nonidet P-40 (Shell Chemical Co.), 1 mM $MnCl_2$, 21 µM [^{3}H]thymidine triphosphate, 854 cpm/pmole, 50 µM poly(A), 5 µM oligo(T) and 5.8 µg/ml viral protein in the form of purified virus. Poly(C)-directed reactions contained the above components except that 50 mM poly(C) replaced poly(A), 5 µM oligo(dG) replaced oligo(T), and 200 µM [^{3}H]deoxyguanosine triphosphate (177 cpm/pmole)replaced [^{3}H]thymidine triphosphate. The source of the polymerase was purified, detergent-disrupted virions, unless otherwise specified. ATA and virus were preincubated for 10 min at 0° in tubes containing an incomplete reaction mix: i.e., a mix with all the components except one, usually poly(A)·oligo(T). The reaction was started after the 10 min period with the addition of the missing component and a shift to 37°. Incorporation of radioactivity into acid-insoluble material was measured after incubating for 10 min at 37°, by spotting either 20 µl or 40 µl onto Whatman 3MM paper discs and washing them as previously described[14].

RESULTS

Time course: Figure 1 shows that when DNA polymerase activity is assayed by the procedure described in Materials and Methods, the rate of incorporation is constant for twenty minutes. In subsequent experiments the incorporation at ten minutes is taken as a measure of this rate.

Stoichiometry: Figure 2 shows the inhibition of RNA-directed DNA synthesis as a function of ATA concentration. Two features of this inhibition are noteworthy. 1.) DNA synthesis falls sharply over a ten-fold change in the concentration of ATA. 2.) The ATA concentration at which inhibition occurs depends on the amount of virus added to the reaction mix; in fact, a linear relationship exists between the amount of viral protein and the ATA concentration necessary to achieve 50% inhibition of poly(A)-directed DNA synthesis.

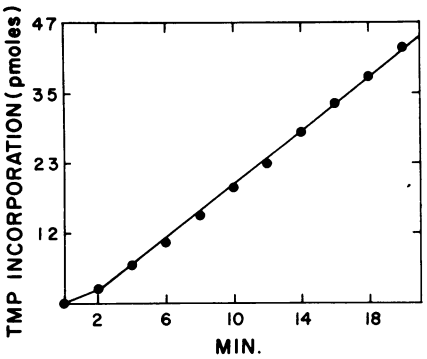

Figure 1. Time course of poly(A)-directed poly(T) synthesis. Reaction mix and procedure are as described in Materials and Methods except the reaction mix had 10.2 µg/ml viral protein and a final volume of 250 µl.

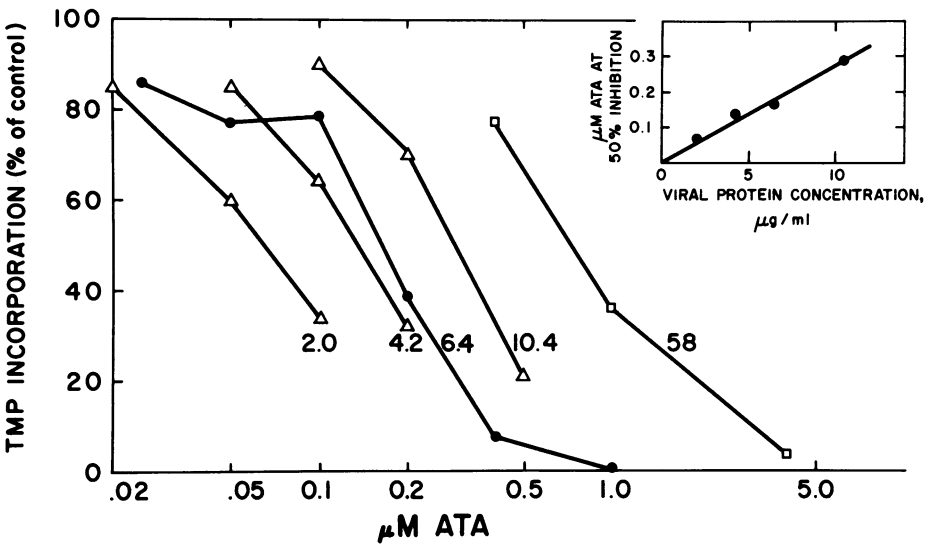

Figure 2. Inhibition of RNA-directed DNA synthesis as a function of ATA concentration and viral protein concentrations. (●—● inhibition of poly(A)-directed poly(T) synthesis in the standard reaction mix by Chelex-ATA. (Δ—Δ) inhibition of poly(A)-directed poly(T) synthesis in the standard reaction mix by untreated Aluminon. (□—□) inhibition of endogenous viral DNA synthesis by untreated Aluminon. Reaction tubes for the viral DNA synthesis contained the same components as the standard mix except there was no poly(A)•oligo(T), only 0.01% Nonidet P-40 and an additional 400 µM each of dCTP, dGTP, and dATP. 100% incorporation represents 16, 33, 57 and 82 pmoles of TMP incorporation in 10 min for the experiments with 2.0, 4.2, 6.4, and 10.4 µg viral protein per ml respectively. The maximum TMP incorporation for the endogenous reaction (58 µg viral protein/ml) was 1.64 pmoles in 60 min. The insert demonstrates the linearity observed between the amount of viral protein and the ATA concentration necessary to achieve 50% inhibition of poly(A)-directed DNA synthesis.

The endogenous polymerase reaction (directed by viral RNA) seems to require proportionately less ATA to reach 50% inhibition. This difference may be attributable to the different conditions used for the two assays. For example, poly(A)-directed poly(T) synthesis is reduced by ten-fold at the low detergent concentration used for the endogenous reaction. This fact suggests that virions are not well-disrupted under these conditions and that binding of ATA to various viral proteins might be altered. However, at detergent concentrations which maximize poly(A)-directed poly(T) synthesis (0.02%-0.10%) the sensitivity of the reaction to ATA is independent of detergent concentration. The much higher nucleoside triphosphate concentration used in the endogenous reaction may also influence the ATA inhibition.

Equal concentrations of crude ATA and Chelex-ATA affect the reaction to the same extent (Fig. 2). Similar results have been obtained with fractions I and II. These data suggest that a component(s) of these ATA preparations inhibits RNA-directed DNA polymerase by interacting stoichiometrically with a viral protein. Since ATA also inhibits poly(A)-directed poly(T) synthesis by purified viral polymerase, the simplest interpretation is that it interacts directly with the polymerase.

Cation effects: To determine if ATA was chelating a necessary co-factor, we examined ATA inhibition of DNA synthesis at various concentrations of Mn^{2+} and Zn^{2+} in the reaction mix. Zn^{2+} was tested because recent evidence suggests that RNA-directed DNA polymerases, like other DNA polymerases, are zinc metalloenzymes[15-17]. Mn^{2+} was tested since it is the cation present in the standard reaction mix. Figure 3 shows that the Mn^{2+} optional concentration is only slightly shifted by ATA. This fact and the low concentrations of ATA needed to inhibit the polymerase reaction, suggest that ATA does not act by binding Mn^{2+}. Similarly, Table 1 shows that ATA inhibition cannot be overcome by adding Zn^{2+} to the reaction mix. (Zn^{2+} itself becomes inhibitory above 0.4 mM,) Thus, ATA apparently does not act by chelating or binding to the poly-

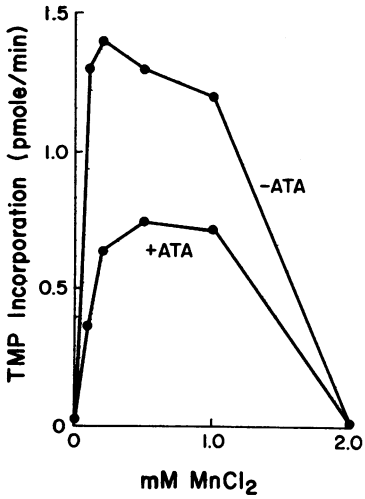

Figure 3. Mn^{2+} optimum. Effect of various $MnCl_2$ concentrations on poly(A)-directed poly(T) synthesis in the presence and absence of 0.5 µM ATA (untreated "Aluminon"). Mix and protocol are as described in Materials and Methods except 8.8 µg/ml of viral protein was present.

Table 1. Effect of various $ZnSO_4$ concentrations on poly(A)-directed DNA synthesis.

Aliquots of the incomplete mix were added to tubes containing $ZnSO_4$ and either water or ATA (untreated "Aluminon"), left at 0° for 10 min and shifted to 37° after the addition of poly(A)·oligo(T). The concentration of viral protein was 5.8 µg/ml. The final concentration of ATA was 0.5 µM.

$ZnSO_4$ concentration (mM)	rate of poly(T) synthesis (pmole TMP per 10 min) +ATA	-ATA
0	0.03	46
0.04	0.23	42
0.1	0.18	53
0.4	0.51	48
1	0.02	1
4	0	0
10	0	0

merase-associated zinc ion.

Table 2 shows that low concentrations of EDTA (up to 100 μM) do not affect the inhibition by ATA. This result and the observations that crude ATA and Chelex-ATA appear to have the same stoichiometry (Fig. 2) and time course (data not given) indicate that the inhibition is not caused by heavy metal ions in the ATA preparation.

Table 2. Effect of low concentrations of EDTA on poly(A)-directed DNA synthesis.

The procedure was that described in the legend of Table 1, except that EDTA replaced $ZnSO_4$ and the final concentration of viral protein was 6.1 μg/ml. The final concentration of ATA was 0.5 μM.

EDTA concentration (μM)	rate of poly(T) synthesis (pmole TMP per 10 min) +ATA	-ATA
0	2.3	71
0.04	1.5	76
0.1	0.82	62
0.4	1.0	72
1	1.2	72
4	1.5	58
10	0.77	87
40	0.30	88
100	0.35	86

Protection by BSA: We examined the protection of the polymerase reaction offered by bovine serum albumin (BSA) in our system. This was determined by adding BSA and ATA to a reaction mix lacking poly(A) and oligo(T). After incubating for 10 min at 0°, the reaction was started with the addition of poly(A)·oligo(T). Five hundred μg/ml BSA results in complete protection of purified polymerase against 1.0 μM ATA. Fifty percent protection was observed with 50 μg/ml BSA and no protection with 5 μg/ml BSA.

Kinetics: Apparent Michaelis-Menten kinetics for the polymerase reaction were obtained for poly(A), poly(C), oligo(T), oligo(dG), TTP and dGTP when the appropriate data were plotted according to Woolf and Hofstee[18]. Figures 4, 5, and 6 demonstrated these kinetics and the effect ATA has on the kinetics for

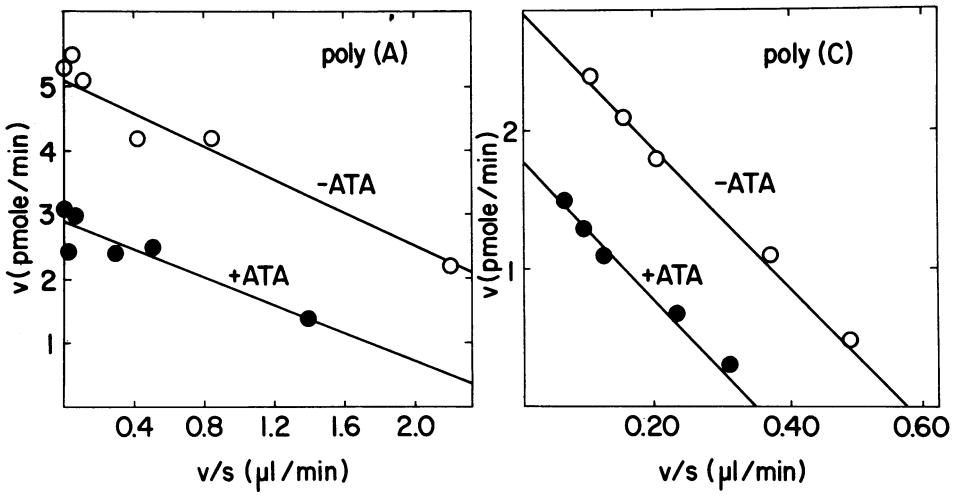

Figure 4. Effect of ATA on the K_m and V_{max} for the template. The poly(A) reaction mixes contained 5.8 µg/ml viral protein and, if present, 0.25 µM Chelex-ATA. The poly(C) reaction mixes contained 10.6 µg/ml viral protein and, if present, 0.3 µM Chelex-ATA.

each of the components listed above. The kinetic constants calculated from the data in Figures 4, 5, and 6 are given in Table 3. The major effect of ATA is an increase in the K_m of the primer; by contrast the K_m does not change for the template or substrate.

Figure 7 shows the relationship between ATA concentration and the K_m for oligo(T). The kinetic constants calculated from these data are given in Table 4. These data support the hypothesis that for the poly(A)-directed reaction the primary effect is the increase in the K_m for oligo(T); the K_m increases 140-fold, while the V_{max} decreases by a factor of 2.4. Furthermore, the

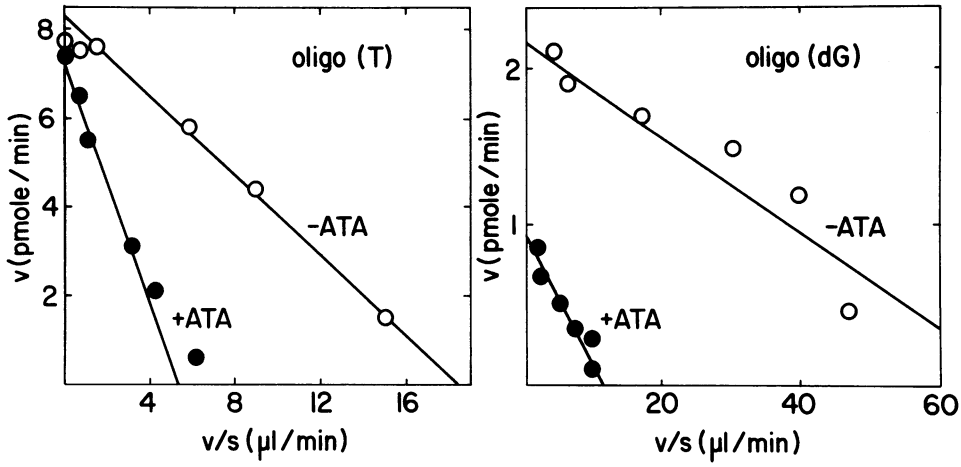

Figure 5. Effect of ATA on the K_m and V_{max} for the primer. The oligo(T) reaction mixes contained 5.8 µg/ml viral protein and if present, 0.25 µM Chelex-ATA. The oligo(dG) reaction mixes contained 9.2 µg/ml viral protein and, if present, 0.3 µM Chelex-ATA.

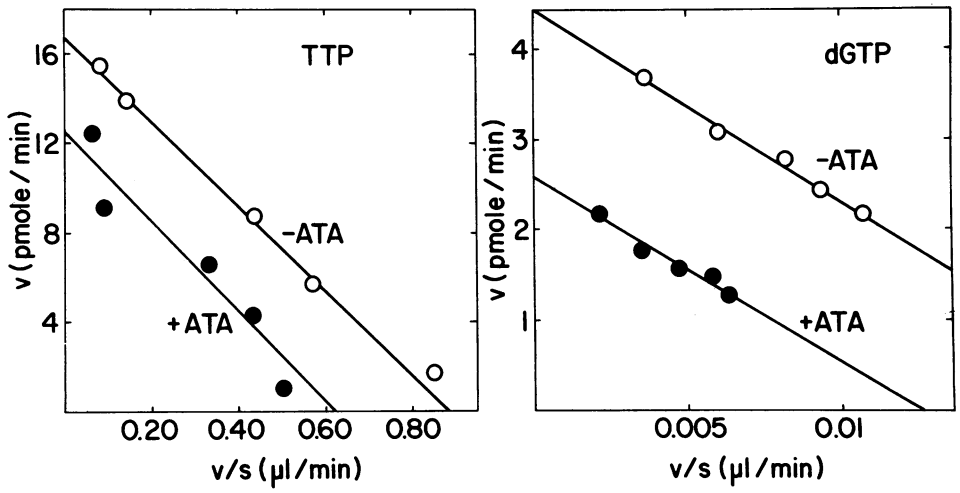

Figure 6. Effect of ATA on the K_m and V_{max} for the substrate. The TTP reaction mixes contained 5.8 µg/ml viral protein and, if present, 0.25 µM Chelex-ATA. The dGTP reaction mixes contained 9.2 µg/ml viral protein and, if present, 0.3 µM Chelex-ATA.

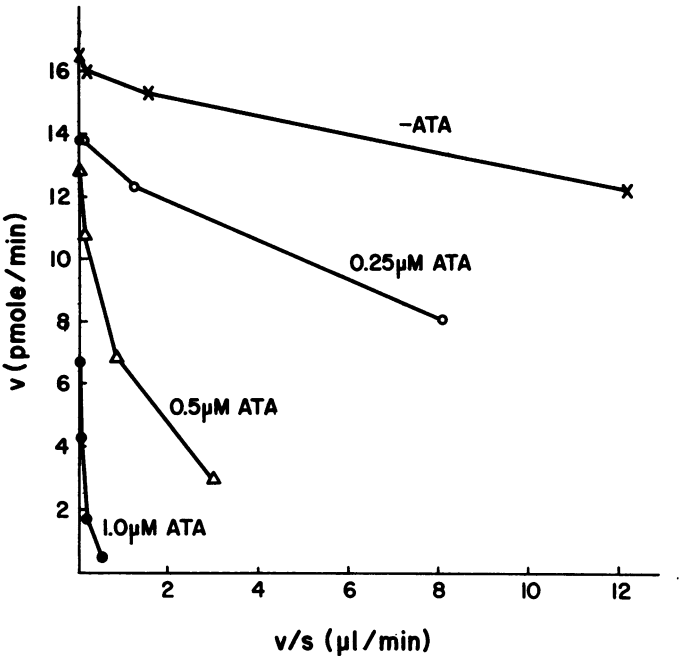

Figure 7. Effect of ATA concentrations on the K_m and V_{max} for oligo(T). Aliquots of the standard reaction mix were added to tubes containing the various amounts of ATA and oligo(T). After 10 min at 0°, the tubes were transferred to 37° and incubated for an additional 10 min. The final concentration of viral protein was 11.8 μg/ml.

Table 3. Kinetic constants calculated from the data in Figures 4, 5 and 6.

Variable	ATA	K_m (μM)*	V_{max} (pmole/min)*
poly(A)	-	1.3 ± 0.3	5.1 ± 0.2
	+	1.1 ± 0.3	2.9 ± 0.2
poly(C)	-	4.9 ± 0.3	2.8 ± 0.1
	+	5.1 ± 0.5	1.8 ± 0.1
oligo(T)	-	0.45 ± 0.01	8.3 ± 0.1
	+	1.4 ± 0.1	7.4 ± 0.1
oligo(dG)	-	0.03 ± 0.004	2.2 ± 0.1
	+	0.08 ± 0.016	0.9 ± 0.1
TTP	-	18.9 ± 0.7	16.7 ± 0.2
	+	20.1 ± 6.7	12.5 ± 1.1
dGTP	-	213 ± 13	4.5 ± 0.1
	+	205 ± 23	2.6 ± 0.1

* Constants and standard deviations were calculated by the method of Wilkinson[19].

Table 4. Kinetics constants calculated from the data in Figure 7.

ATA (μM)	K_m[a] (μM)	V_{max}[a] (pmole/min)
0	0.33 ± 0.05	16.1 ± 0.2
0.25	0.71 ± 0.09	13.7 ± 0.2
0.50	6.4 ± 2.9	12.2 ± 1.0
1.00	45. ± 18.	6.8 ± 0.6

[a] Constants and standard deviations were calculated by the method of Wilkinson[19].

relative linearity of the lines in Figures 5 and 7 and the continual increase of K_m with ATA concentration (Fig. 7) show that, to a first approximation, all polymerase molecules are equally affected by the presence of ATA (see Discussion).

Time course experiments were done at various concentrations of Chelex-ATA, fraction I, fraction II, and untreated Aluminon. Samples were taken every 15 seconds and the mixes were incubated at 32°. In all experiments the course of incorporation after addition of ATA back-extrapolated to the incorporation level at the time of ATA addition (data not shown). Thus, this experiment, unlike similar experiments with RNA polymerases[8-10], did not detect any synthesis corresponding to the completion of DNA molecules which were being synthesized at the time of addition of ATA (runoff synthesis). This result is compatible with recent evidence that DNA polymerases, unlike RNA polymerases, are not pocessive enzymes *in vitro*[22,23]. However, our interpretation of this experiment is compromised by our lack of knowledge of how much runoff synthesis to expect.

DISCUSSION

Several lines of evidence presented in this report lead to the conclusion

that ATA inhibits RNA-directed DNA polymerase by interacting with the enzyme itself. First, we have ruled out several possible indirect mechanisms of inhibition. We have shown that the ATA preparation does not inhibit the polymerase reaction by sequestering Mn^{2+} or Zn^{2+}. Furthermore, ATA inhibition differs from inhibition of Rous sarcoma virus DNA polymerase by N-methyl isatin β-thiosemicarbazone in that it does not involve contaminating heavy metal ions[21]. The detergent optimum for the reaction is also unaffected by the presence of ATA. Second, the concentration of ATA necessary to inhibit the polymerase reaction is directly proportional to the concentration of the viral protein but not to the concentration of template, substrate, or primer. These observations are consistent with the notion of a stoichiometric interaction between ATA and the polymerase or some other viral protein. The fact that ATA inhibits our purified polymerase suggests that it interacts directly with the polymerase. However, since we do not know the degree of purity of the purified polymerase we cannot rule out the possibility that ATA inhibits by interacting with another viral protein. Indeed, the fact that ATA binds to BSA[8,20] suggests that it can bind to many proteins. However, the kinetics of the polymerase reaction also provide evidence for some specificity in the ATA:polymerase interaction; namely, that ATA affects the binding of polymerase to its primer molecule. In this respect, our results are similar to previous results which have shown that ATA lowers the affinity of Qβ replicase, E. coli RNA polymerase and lac repressor for nucleic acids [8,9]. Thus, the simplest explanation for its inhibition is that it interacts directly with the polymerase.

If ATA competes for the nucleic acid binding site on the polymerase, then the reaction should saturate at a higher nucleic acid concentration in the presence of ATA. Although the original Michaelis-Menten model may not be appropriate for polymerases, apparent Michaelis-Menten kinetics were obtained for poly(A), poly(C), oligo(T), oligo(dG), TTP and dGTP. This behavior allows us to characterize the reaction with two parameters, an apparent V_{max} and an

apparent K_m. Inhibition by ATA decreased the V_{max} for all of the above templates, primers, and substrates and increased the K_m for only oligo(T) and oligo(dG). Since both the V_{max} and K_m for oligo(T) and oligo(dG) are changed by ATA this inhibition is classified as a "mixed" type[18]. These results indicate that ATA affects the DNA primer binding site(s) of the Moloney leukemia virus RNA-directed DNA polymerase.

The fact that the K_m of the primer increases as the ATA concentration is raised suggests that ATA binds to the polymerase reversibly and reduces the binding of the primer[18]. If ATA bound irreversibly to the polymerase, the inhibited enzyme preparations would be mixtures of permanently inhibited and uninhibited molecules. In that case, the lines in Figure 5 would be biphasic, one section representing the inhibited molecules and the other the uninhibited. We did not observe biphasic kinetics, although there is some curvature of the lines in Figure 5 at high ATA concentrations.

Previous reports dealing with ATA inhibition of RNA polymerase action concluded that, at low concentrations, ATA inhibits initiation of the polymerase reaction. These conclusions are inferred in part from the fact that RNA polymerase was less sensitive to ATA after the polymerization reaction had begun[8]. This analysis assumed that the RNA polymerase is a processive enzyme; that is, one which normally completes an RNA molecule before dissociating from the template and becoming available to a new template molecule. If the DNA polymerase were also a processive enzyme, one might expect that ATA might be able to inhibit the initiation (priming) of new DNA chains without affecting the elongation of partially completed molecules. However, the time course of incorporation before and after addition of ATA showed that incorporation ceased immediately upon addition of the inhibitor. This result is compatible with recent evidence that DNA polymerases, at least under _in vitro_ conditions, are not processive enzymes[22,23].

ACKNOWLEDGMENTS

The authors would like to thank Victoria Onorato for dedicated technical assistance, Dr. L.M.S. Chang for communication of her results before publication, and Drs. E. Sulkowski and S.K. Arya for critical reading of the manuscript. This work was supported by grants CA-13479 and CA-14801 from the National Cancer Institute.

Graduate Faculty in Microbiology, State University of New York at Buffalo, (Roswell Park Division), Buffalo, NY 14263, USA.

REFERENCES

1 The abbreviations used are: ATA, aurintricarboxylic acid; BSA, bovine serum albumin; TTP, thymidine triphosphate; dGTP, deoxyguanosine triphosphate; and EDTA, ethylenediaminetetracetic acid.
2 Grollman, A.P. and Stewart, M.L. (1968) Proc. Nat. Acad. Sci. U.S.A. 61, 719-725
3 Stewart, M.L., Grollman, A.P. and Huang, M.T. (1971) Proc. Nat. Acad. Sci. U.S.A. 68, 97-101
4 Huang, M.T. and Grollman, A.P. (1972) Mol. Pharmacol. 8, 111-127
5 Siegelman, F. and Apirion, D. (1971) J. Bacteriol. 105, 902-907
6 Webster, R.E. and Zinder, N.D. (1969) J. Mol. Biol. 42, 425-439
7 Wilhelm, J.M. and Haselkorn, R. (1970) Proc. Nat. Acad. Sci. U.S.A. 65, 388-394
8 Blumenthal, T. and Landers, T.A. (1973) Biochem. Biophys. Res. Commun. 55, 680-688
9 Liao, L.L., Horwitz, S.B., Huang, M.T., Grollman, A.P., Steward, D. and Martin, J. (1975) J. Med. Chem. 18, 117-120
10 Hunt, D.M. and Wagner, R.R. (1975) Abstracts of the Annual Meeting of the American Society for Microbiology, p. 251
11 Leis, J.P. and Hurwitz, J. (1972) J. Virol. 9, 130-142
12 Manly, K.F. (1975) Anal. Biochem. 63, 491-500
13 Faras, A.J., Taylor, J.M., McDonnell, J.P., Levinson, W.E. and Bishop, J.M. (1972) Biochem. 11, 2334-2351
14 Manly, K.F. (1974) J. Virol. 13, 305-311
15 Springgate, C.F., Mildvan, A.S., Abramson, R., Engle, J.L. and Loeb, L.A. (1973) J. Biol. Chem. 248, 5987-5993
16 Poiesz, B.J., Battula, N. and Loeb, L.A. (1974) Biochem. Biophys. Res. Commun. 56, 959-964
17 Auld, D.S., Kawaguchi, H., Livingston, D.M. and Vallee, B.L. (1974) Biochem. Biophys. Res. Commun. 57, 967-972
18 Dixon, M. and Webb, E.C. (1974) Enzymes, 2nd Edn. pp. 63-80, Academic Press, New York
19 Wilkinson, G.N. (1961) Biochem. J. 80, 324-332
20 Lindenbaum, A. and Schubert, J. (1956) J. Phys. Chem. 60, 1663-1665
21 Levinson, W., Faras, A., Woodson, B., Jackson, J. and Bishop, J.M. (1973) Proc. Nat. Acad. Sci. U.S.A. 70, 164-168
22 Chang, L.M.S. (1975) J. Mol. Biol. 93, 219-235
23 McClure, W.R. and Jovin, T.M. (1975) J. Biol. Chem. 250, 4073-4080