Inhibition by Aurintricarboxylic Acid and Polyethylene Sulfonate of RNA Transcription of Vesicular Stomatitis Virus

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Received for publication 13 May 1975

The in vitro activity of the ribonucleoprotein-dependent RNA transcriptase of vesicular stomatitis virions was found to be completely inhibited by low concentrations of aurintricarboxylic acid (ATA) and polyethylene sulfonic acid (PES) when these inhibitors were added before the start of the RNA polymerase reaction. However, if RNA synthesis was allowed to occur before ATA or PES was added, RNA synthesis continued for a short time (10 min or less) in the presence of either inhibitor at a concentration which completely inhibited uninitiated enzyme. The ability to continue to synthesize RNA in the presence of ATA or PES only developed if all four nucleoside triphosphates were present during the preincubation period prior to the addition of the inhibitors. The protection was apparently not due to the released products of RNA polymerization. The results are interpreted as indicating that ATA and PES probably inhibit some reaction other than elongation of RNA chains, and that this reaction might be one involved at or near initiation sites.

Vesicular stomatitis virus (VSV) is a single-stranded RNA virus belonging to the rhabdovirus group. The virion contains an RNA-dependent RNA polymerase (1) which, when assayed in vitro, synthesizes RNA complementary to the virion RNA (1, 2, 5). mRNA transcribed in vivo is also complementary to virion RNA (13, 19). Both the product of the in vitro virion RNA polymerase reaction and mRNA isolated from VSV-infected cells can be translated in vitro, yielding viral-specific polypeptides (7, 8, 16). Thus, it appears that one function of the virion RNA polymerase is synthesis of mRNA. Emerson and Wagner (10) have shown that virions can be separated into two fractions by treatment with Triton X-100 and high salt followed by ultracentrifugation. The pellet fraction contains RNA complexed with N protein and the supernatant fraction contains proteins G, M, L, and NS (10-12, 23). It appears that the nucleocapsid N-protein-RNA complex functions as a template and the L and NS proteins are both essential for transcription but that G and M proteins are not required (11, 12, 21). The transcriptase requires VSV ribonucleoprotein, or ribonucleoprotein from a closely related virus, as a template and will not function with deproteinized viral RNA (3, 10).

A useful tool for the in vitro study of this complex system would be the availability of inhibitors which selectively affect initiation of RNA synthesis or chain elongation. In this preliminary study we report that aurintricarboxylic acid, ammonium salt (ATA), and polyethylene sulfonate, sodium salt (PES), both of which selectively inhibit initiation of RNA synthesis by other RNA polymerases (6, 9, 17), might have a somewhat similar effect on the VSV transcriptase.

(This work was presented in part at the 75th Annual Meeting of the American Society for Microbiology, New York City, April 27-May 2, 1975.)

MATERIALS AND METHODS

Viruses and cell cultures. The strain of VSV Indiana used was one originally obtained from the U.S. Agricultural Research Center, Beltsville, Md. (22). Virus was grown at 31 C in confluent layers of BHK-21, clone 13 cells in 75-cm² Falcon flasks in the presence of 90% BHK-21 medium and 10% tryptose phosphate broth. Cultures were infected with cloned virus at a multiplicity of 1 PFU/cell. Released virions were harvested 18 h after infection and were free of defective virions.

Purification of virions. Virions were purified by differential and rate zonal centrifugation as previously described (10), except that the sucrose gradients contained 1 M NaCl and 1 mM EDTA. The virus obtained by this procedure was further purified by equilibrium centrifugation for 16 h at 35,000 x g on 0 to 40% (wt/wt) potassium tartrate-0 to 20% (vol/vol) glycerol gradients which contained 10 mM Tris-hydrochloride, pH 7.4, and 1 mM EDTA. The viral band was removed from the tartrate gradients, diluted with reticulocyte standard buffer (RSB; 10⁻² M
glycerol-50%ride, VOL. virions glycerol, in One min (RSB-15% glycerol). tubes, which then out glycerol-1 RSB-15% lengths appropriate mixture In assay i.e., ATP, solutions, mock x nucleoside phates); RNA/ml). The method RNA/ml). (U-6812 lot no. 34362-RCA-43J), unfraccionated by molecular weight range, was kindly provided by Upjohn Co., Kalamazoo, Mich. [3H]UTP (16 to 21 C/mmol) was obtained from Schwarz/Mann, Orangeburg, N.Y., and Amersham/Searle, Arlington Heights, Ill. Nitrocellulose filters (0.45 μm pore size) were purchased from Millipore Corp., Bedford, Mass.

Solutions. Both PES and ATA were dissolved in 10 mM Tris-hydrochloride, pH 8.0. ATA was always made up immediately before use and kept in the dark.

RESULTS

PES and ATA inhibit the VS virion transcriptase. The effects on transcription of adding various concentrations of ATA or PES to the viral RNA polymerase assay mix immediately before incubation at 31 C are shown in Fig. 1. Concentrations of 10 to 25 μM ATA (Fig. 1A) and 1 to 10 μg of PES/ml (Fig. 1B) resulted in more or less complete inhibition of the RNA transcriptase. The data indicate that the degree of inhibition was concentration dependent. The degree of inhibition observed was somewhat dependent on viral protein concentration. For example, if the virus concentration was increased by a factor of five, then the results shown in Fig. 1C (ATA) and Fig. 1D (PES) were obtained. In subsequent experiments, the protein concentration was kept in the range 25 to 45 μg of viral protein/ml of complete assay mix to insure more or less complete inhibition of RNA synthesis when 25.0 μM ATA or 10.0 μg of PES/ml was added before the start of the RNA transcriptase reaction.

Effect of preincubation of the complete assay mix before addition of each inhibitor. If a compound inhibits initiation of RNA synthesis but has no effect on RNA chain elongation, then chains initiated before addition of the inhibitor should continue to be elongated after the inhibitor is added. Since no further initiation should occur after the addition of the inhibitor, RNA synthesis would cease after all preinitiated chains had been completed.

To determine whether the inhibitors act on initiation or elongation of RNA chains the complete polymerase assay mix was preincubated for various times at 31 C to allow initiation to occur. The tubes were then placed on ice to halt the reaction; ATA, or PES, or buffer was then added and the subsequent time course of [3H]UTP incorporation at 31 C into acid-insoluble material was followed. The zero time point for this, and for all other experiments in which preincubations were carried out, is taken to be the start of the second incubation at 31 C. The results (Fig. 2) indicate that, if the enzyme was allowed to start synthesizing RNA before ATA or PES was added, then synthesis continued for a short time (10 min or less) after addition of ATA or PES at concentrations which completely inhibited nonpreincubated samples. The amount of RNA synthesis observed after addition of ATA or PES was a function of the time of preincubation. In this experiment a 15-min preincubation period without inhibitor...
FIG. 1. Effect of ATA and PES on RNA transcription by VS virions. Virus was treated at 0 C with 2x high-salt solubilizer, RSB-20% glycerol, and prereaction solution. To 1.0 ml of the complete assay mix was added 5 µl of 10 mM Tris-hydrochloride, pH 8.0, or 5 µl of solutions of ATA or PES of the appropriate concentrations. Samples (0.1 ml) of the assay mix were then put into test tubes and incubated for the desired length of time at 31 C, and incorporation of [3H]UMP into acid-insoluble material was determined. The concentration of ATA or PES indicated in the figure are final concentrations. All values plotted are the average of duplicate determinations; µg of protein/ml of complete assay mix = 32 (A); 60 (B); 160 (C); 160 (D).
To test the effect of preincubation with initiator nucleoside triphosphates, the VSV transcriptase was preincubated for 15 min with complete assay mix except that some (or all) nucleoside triphosphates were omitted. At the end of the preincubation period, the various assay mixes were placed on ice and any missing nucleoside triphosphates were added; ATA, or PES, or buffer was then added, and the subsequent time course of $[^3H]$UMP incorporation at 31°C was followed.

Figure 3 compares the time course, in the absence of ATA or PES, of $[^3H]$UMP incorporation during the second incubation at 31°C of enzyme which was preincubated (A) in the presence of all four nucleoside triphosphates or (B) in the absence of all four nucleoside triphosphates; the missing triphosphates in (B) were added at the end of the preincubation period. Preincubation for 15 min at 31°C in the absence of nucleoside triphosphates apparently does not

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**FIG. 2.** Effect of preincubating complete VSV polymerase assay mix at 31°C before adding ATA or PES. Complete polymerase assay mix was preincubated at 31°C for 0, 5, 10, 15 or 30 min. The vials (each containing 1.0 ml of the preincubated assay mix) were then placed on ice, and 5 μl of 10 mM Tris-hydrochloride, pH 8.0, or 5 μl of 5 mM ATA (final concentration 25 μM), or 5 μl of 2 mg of PES/ml (final concentration 10 μg/ml) was then added. Samples (0.1 ml) were pipetted into test tubes, and the subsequent time course of incorporation of $[^3H]$UMP at 31°C into acid-insoluble material was determined. The incorporation at zero time (i.e., the start of the second 31°C incubation) was subtracted from zero min and subsequent time points and varied from 232 counts/min (no preincubation) to 16,636 counts/min (30-min preincubation). All time points are the average of duplicate determinations. Protein concentration = 33 μg/ml of complete polymerase assay mix. Duration of preincubation period: □, 0 min; Δ, 10 min; ●, 15 min; ○, 30 min.

**Effect of preincubation in the absence of certain nucleoside triphosphates.** If, as we suppose, the transcriptase inhibitors affect only initiation rather than chain elongation, then it is conceivable that preincubation of the template-enzyme complex with initiator nucleoside triphosphates might prevent transcriptase inhibition by ATA or PES. We hoped this way to identify the specific nucleoside triphosphate(s) required for initiation and for subsequent chain elongation in the presence of inhibitors.

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**FIG. 3.** Effect of preincubation for 15 min at 31°C in the presence or absence of nucleoside triphosphates on subsequent nucleotide incorporation at 31°C in the presence of all four nucleoside triphosphates. Complete polymerase assay mix or polymerase assay mix minus all four nucleoside triphosphates was preincubated for 15 min at 31°C. The assay mixes were then put on ice and any missing nucleosides were added. Subsequent incorporation of $[^3H]$UMP into acid-insoluble material at 31°C was then monitored. Zero-minute time point represents the start of the second incubation at 31°C. All points are the average of duplicate determinations. (A) Preincubated with all four nucleoside triphosphates (●); (B) preincubated in the absence of all four nucleoside triphosphates (○).
result in any inactivation of the enzyme. Figure 3 shows that the final rate of incorporation is the same. However, absence of all four nucleoside triphosphates during preincubation results in a greater lag phase during the second 31 C incubation than is observed with enzyme preincubated in the presence of all four nucleoside triphosphates. Preincubation in the presence of any combination of three nucleoside triphosphates, followed by addition of the missing nucleoside triphosphate at the end of the preincubation period, resulted in kinetics of [3H]UMP incorporation in the second incubation at 31 C identical to those shown in (B) of Fig. 3. That is, the lag was not reduced, and the enzyme was not inactivated, by preincubation with any combination of three nucleoside triphosphates.

The results shown in Table 1 indicate that the VSV transcriptase-template complex must be preincubated prior to addition of ATA or PES with all four nucleoside triphosphates if any nucleotide incorporation is to be observed in the presence of ATA or PES. No combination of three nucleoside triphosphates is sufficient for the transcriptase system to develop any significant ability to incorporate [3H]UMP in the presence of the inhibitors.

Is the protection observed after preincubation due to the presence of end products of the RNA polymerase reaction? A possible explanation of the above results is that during RNA synthesis, prior to addition of inhibitor, products are released (e.g., pyrophosphate, RNA) and the presence of these products protects the enzyme and allows RNA synthesis to continue for a short time after the addition of ATA or PES.

To test the hypothesis that preincubation reaction products counteract the effect of ATA and PES on RNA synthesis, complete assay mix was incubated at 31 C for 15 min, initiated enzyme and template were removed by ultracentrifugation (150,000 × g for 150 min), and then the supernatant from this centrifugation was added to a complete assay mix. If the released end products of RNA polymerization protect the transcriptase from ATA or PES, then complete assay mix plus preincubated supernant should be able to synthesize RNA in the presence of ATA or PES without any incubation at 31 C prior to addition of the inhibitor.

The data in Table 2 show that there was no significant incorporation when supernatant from either an unincubated or an incubated mix was added to complete assay mix and ATA or PES was added immediately. Thus, it appears that the end products present in the supernatant from the incubated mix did not result in any significant protection of the enzyme from the effects of ATA and PES. If complete assay mix plus either unincubated or incubated supernatant was preincubated at 31 C prior to the addition of ATA or PES, then significant incorporation in the presence of the inhibitor was observed. Therefore, the soluble end products in the incubated supernatant did not prevent to any great extent the ability of the viral RNA polymerase to synthesize RNA in the presence of ATA or PES.

Effect of increasing the concentration of UTP in the assay mix. The concentration of UTP used in the complete assay mix in the experiments described above (9.5 × 10⁻⁸ M) is somewhat less than the apparent Kₘ for UTP in this assay (apparent Kₘ = 19 × 10⁻⁶ M; unpublished observations). Experiments were therefore carried out under conditions in which UTP was not limiting (66.5 × 10⁻⁸ M UTP) to ascertain whether the ability to synthesize RNA in the presence of PES or ATA is influenced by the concentration of UTP.

The results (Table 3) show that, in a 30-min incubation at 31 C in the absence of inhibitors, the amount of UMP incorporation was ~ three times greater in the presence of 66.5 × 10⁻⁸ M
TABLE 2. Effect of supernatant fractions from preincubated or nonincubated assay mixes on nucleotide incorporation by complete assay mix in the presence of ATA or PES

<table>
<thead>
<tr>
<th>Components of assay mix</th>
<th>Preincubation before addition of inhibitor</th>
<th>Inhibitor</th>
<th>$[^{3}P]UTP$ incorporation* (counts/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete + unincubated SN</td>
<td>no</td>
<td></td>
<td>5,566</td>
</tr>
<tr>
<td>Complete + unincubated SN</td>
<td>no</td>
<td>ATA</td>
<td>61</td>
</tr>
<tr>
<td>Complete + unincubated SN</td>
<td>no</td>
<td>PES</td>
<td>-35</td>
</tr>
<tr>
<td>Complete + incubated SN</td>
<td>no</td>
<td></td>
<td>6,318</td>
</tr>
<tr>
<td>Complete + incubated SN</td>
<td>no</td>
<td>ATA</td>
<td>44</td>
</tr>
<tr>
<td>Complete + incubated SN</td>
<td>no</td>
<td>PES</td>
<td>-114</td>
</tr>
<tr>
<td>Complete + unincubated SN</td>
<td>yes</td>
<td></td>
<td>9,936</td>
</tr>
<tr>
<td>Complete + unincubated SN</td>
<td>yes</td>
<td>ATA</td>
<td>1,347</td>
</tr>
<tr>
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<td>yes</td>
<td>PES</td>
<td>1,325</td>
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<td>yes</td>
<td></td>
<td>9,135</td>
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<tr>
<td>Complete + incubated SN</td>
<td>yes</td>
<td>ATA</td>
<td>1,126</td>
</tr>
<tr>
<td>Complete + incubated SN</td>
<td>yes</td>
<td>PES</td>
<td>851</td>
</tr>
</tbody>
</table>

* Two complete polymerase assay mixes were prepared. One was incubated at 31°C for 15 min ($[^{3}P]UTP$ incorporation = 6,854 counts/min per 0.1 ml) and the other was kept on ice. Both were then centrifuged at 150,000 × g for 15 min, and the supernatant fractions were collected. The supernatant from the incubated mix (incubated SN) contained 3,575 trichloroacetic acid-insoluble counts/min per 0.1 ml. A third complete assay mix was prepared from the same preparation of virus. 0.5-ml samples of this were diluted with 0.5 ml of incubated SN or unincubated SN. The 1.0-ml mixes were then either preincubated at 31°C for 15 min and then put on ice or not preincubated. Tris-hydrochloride, pH 8.0 (10 mM), or ATA (25 μM), or PES (10 μg/ml) was then added, and subsequent incorporation at 31°C of $[^{3}P]UMP$ into acid-insoluble material was monitored.

TABLE 3. Effect on RNA synthesis of increasing the concentration of UTP in complete VS transcription assay mix

<table>
<thead>
<tr>
<th>UTP (× 10-6 M)</th>
<th>Inhibitor</th>
<th>Unincubated assay mix (pmol of UMP)</th>
<th>Preincubated assay mix (pmol of UMP)</th>
</tr>
</thead>
<tbody>
<tr>
<td>9.5</td>
<td>ATA</td>
<td>38.8</td>
<td>42.6</td>
</tr>
<tr>
<td>9.5</td>
<td>PES</td>
<td>0.3</td>
<td>7.3</td>
</tr>
<tr>
<td>9.5</td>
<td></td>
<td>0.6</td>
<td>5.8</td>
</tr>
<tr>
<td>66.5</td>
<td>ATA</td>
<td>109.7</td>
<td>138.3</td>
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<tr>
<td>66.5</td>
<td>PES</td>
<td>1.4</td>
<td>10.8</td>
</tr>
<tr>
<td>66.5</td>
<td></td>
<td>0.3</td>
<td>6.0</td>
</tr>
</tbody>
</table>

* One set of 1-ml samples of complete assay mix containing either 9.5 × 10-6 M UTP or 66.5 × 10-6 M UTP was preincubated at 31°C for 15 min and then placed on ice. Buffer or 25 μM ATA or 10 μg of PES/ml was then added, and subsequent incorporation at 31°C was monitored ("preincubated assay mix"). Another set of 1-ml samples of complete assay mix was not preincubated at 31°C but was kept on ice. Buffer, or 25 μM ATA, or 10 μg of PES/ml was added to these, and incorporation at 31°C was monitored ("unincubated assay mix"). Viral protein concentration = 38 μg/ml of complete assay mix.

* Incorporation per 0.1-ml sample at 31°C in first 30 min after addition of inhibitor or buffer.

** Incorporation irrespective of the UTP concentration. Addition of ATA or PES to preincubated complete assay mixes, followed by incubation at 31°C, resulted in incorporation at 31°C in the presence of the inhibitor. The amount of this incorporation was approximately the same whether the UTP concentration was 9.5 × 10-6 or 66.5 × 10-6 M.

** DISCUSSION

We have shown that in vitro transcription by the VS virion RNA polymerase is inhibited by low concentrations of ATA and PES, or, conceivably, by some impurity present in our preparations of these compounds (6). If the inhibitors are added before the start of the polymerase reaction, 25 μM ATA or 10 μg of PES/ml almost completely inhibits the polymerase as long as the protein concentration is not too high. However, if the virus is allowed to synthesize RNA prior to the addition of inhibitors, RNA synthesis continues after the addition of PES or ATA. Such synthesis occurs only for a short time (10 min or less, Fig. 2) and then ceases. These data are consistent with a model in which RNA chains cannot be initiated in the presence of the inhibitors, but preinitiated RNA synthesis is continued. **
chains can be elongated. The kinetics observed can be explained by presumed cessation of RNA synthesis once all preinitiated chains are completed.

A possible explanation for the observation that approximately 15 min of RNA synthesis is required to develop the full potential of the polymerase to synthesize RNA after subsequent addition of inhibitor could be based on a requirement for RNA synthesis to take place for this length of time for the genome to be fully loaded with transcriptase. This is supported to some extent by the fact that, in the absence of inhibitors, there is a lag of approximately 15 min before the rate of transcription is maximal. This lag is probably not due to a requirement for an ATP-dependent reaction, such as a phosphokinase-mediated phosphorylation, to occur before the transcriptase is fully active (24) as preincubation for 15 min at 31°C with ATP or with any combination of three nucleoside triphosphates does not abolish the lag (unpublished observations). Although the above observations could also be interpreted to mean that end products of RNA synthesis result in the transient protection against ATA or PES, this appears unlikely as the released end products of incubation at 31°C for 15 min, freed of initiated enzyme and nucleocapsid by centrifugation, were not able to produce this protection.

The data from the experiments in which two concentrations of UTP were used are not inconsistent with the hypothesis that ATA and PES preferentially inhibit some reaction involved in initiation rather than elongation. It was observed that, in the absence of inhibitors, enzyme exposed to 65.5 x 10^-4 M UTP made ~ three times more RNA than that exposed to 9.5 x 10^-4 M UTP. This could be due to the rate of chain elongation being dependent on UTP concentration under these circumstances. However, in the presence of inhibitors, preincubated polymerase made approximately the same amount of RNA irrespective of UTP concentration. A possible explanation is that the number of initiated chains in the reaction mix is not greatly affected by the UTP concentration and so the amount of elongation after addition of ATA or PES is approximately equivalent at both UTP concentrations.

Thus, it appears that for the transcriptase to be able to make RNA in the presence of ATA or PES, prior initiation of RNA synthesis is necessary. The data are consistent with elongation of preinitiated chains and failure to initiate new chains in the presence of ATA or PES. Unfortunately, the amount of RNA synthesis after addition of inhibitor is so low, and the apparent $K_a$ for ATP so high (4), that the use of $\gamma^3$P-labeled purine triphosphates to detect whether or not initiation was inhibited was not feasible. Furthermore, the probability that the 5'-terminal of the RNA is methylated and blocked (20) complicates any such analysis. If ATA or PES inhibit the enzyme prior to binding to the template and/or the binding of the first nucleoside triphosphate, then preincubation with at least one of the combinations of three nucleoside triphosphates would be expected to result in some protection. However, none was observed. This could imply that at least some polymerization is necessary before the polymerase develops any degree of resistance to the inhibitors. This observation with the VSV polymerase thus differs from that of Kondo and Weissmann (17) and Blumenthal and Landers (6) who examined the effects of PES and ATA, respectively, on Q8 RNA replicase and found that preincubation with an initiating nucleoside triphosphate was sufficient for an appreciable amount of protection against the effects of ATA or PES to be developed. An alternative explanation of our results could be that, when preincubations of the VSV transcriptase are carried out in complete assay mix, there is more than one polymerase per transcription unit at the time at which inhibitor is added. If one nucleoside triphosphate is missing, however, there can be only one polymerase per transcription unit as it cannot progress past the initiation site and thus leave the site available for further enzyme molecules to initiate. Thus, if the number of initiated chains is reduced by, for instance, a factor of 10, our experiments would not be sensitive enough to detect RNA synthesis in the presence of inhibitor.

From these preliminary studies it appears that ATA and PES, or some component(s) present in our preparations of these compounds, inhibit RNA synthesis to a far greater extent when added prior to the start of the reaction than they do when added during the course of the reaction. A possible explanation of our results is that the inhibitory effect of these compounds is due to their acting at, or near to, an initiation site. However, we cannot completely exclude an effect on chain elongation and/or chain termination.

At the moment we cannot propose a mechanism for the action of ATA or PES. ATA affects a variety of reactions other than RNA synthesis (2, 14) and binds to a large number of proteins (14). Such a large, charged molecule as polyethylene sulfonate is also likely to have a variety of effects rather than a specific action. Nor can we exclude at this time the possibility of indirect
ACKNOWLEDGMENTS

This research was supported by Public Health Service grant AI-11112 from the National Institute of Allergy and Infectious Diseases, by grant BMS72-02229 from the National Science Foundation, and by grant VC-88 from the American Cancer Society.

We are grateful to Suzanne U. Emerson and J. Thomas Parsons for many useful discussions and suggestions.

LITERATURE CITED


