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Inhibition of Mammalian Polyadenylate Polymerase by 2-Aza-1,N^6-etheno-adenosine Triphosphate[1]

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SUMMARY

2-Aza-1,N^6-etheno-adenosine triphosphate (aza-ϵATP), a fluorescent analog of adenosine triphosphate, significantly inhibits polyadenylate [poly(A)] polymerase of bovine lymphosarcoma and calf thymus, with 50% inhibition at 200 μM (in the presence of an equal concentration of adenosine triphosphate). Calf thymus RNA polymerases II and III are inhibited 32 and 20%, respectively, by a 3.8-fold excess of aza-ϵATP; DNA polymerase α is not inhibited. The inhibition of poly(A) polymerase by aza-ϵATP appears to be competitive with adenosine triphosphate; incorporation of aza-ϵATP is not observed. Polymers of 2-aza-1,N^6-etheno-adenosine monophosphate are used as primers, but poorly.

1,*N*-Etheno-adenosine triphosphate and 9-β-D-arabinofuranosyladenine triphosphate are poor inhibitors of poly(A) polymerase; adenosine diphosphate is ineffective. Deoxyadenosine triphosphate inhibits to the same extent as aza-ϵATP, while other naturally occurring nucleotides inhibit poly(A) polymerase to varying degrees, with deoxynucleoside triphosphates more potent than ribonucleoside triphosphates.

Inhibition of poly(A) polymerase by naturally occurring nucleoside triphosphates suggests that nucleotides may regulate the enzyme *in vivo*; inhibition by the fluorescent analog aza-ϵATP suggests that this compound may be useful in elucidating poly(A) metabolism in both normal and neoplastic cells.

INTRODUCTION

aza-ϵATP[3] (26) is an interesting synthetic analog of ATP, since the presence of the etheno group renders the analog fluorescent, making it potentially useful in histological studies (20). The etheno group may also protect the corresponding nucleoside from the deamination occurring *in vivo* with other adenosine analogs (15). Aza-ϵ adenosine is cytotoxic in a rat mammary tumor tissue culture line but not in HeLa and Glioma 26 tissue culture lines (22). In analogy with the arabinosyl nucleoside, it may be converted to the triphosphate as the active form.

Nucleotide analogs interrupt the transfer of information from DNA to RNA to protein by inhibiting synthesis of nucleic acids or one of their nucleotide constituents. One of the steps in the transfer of information is the addition of a poly(A) tract to the 3'OH end of heterogeneous nRNA (11, 13, 14). This step occurs posttranscriptionally (2) and appears to be important in RNA metabolism since most mRNA species contain poly(A) (12). Mn^{2+}- and Mg^{2+}-dependent poly(A) polymerases have been isolated from mammalian tissues (19, 24, 25), and one or both may be responsible for the posttranscriptional addition of poly(A).

In our laboratory, a number of the enzymes thought to be involved in information transfer have been isolated and extensively purified, from both calf thymus and bovine lymphosarcoma: DNA polymerase α (6, 23), RNA polymerase II (7, 8), RNA polymerase III (5, 7, 8), and Mn^{2+}-dependent poly(A) polymerase (9). In this paper, we examine the effect of aza-ϵATP on these enzymes and compare inhibition of poly(A) polymerase with the effect of natural nucleotides and other ATP analogs.

MATERIALS AND METHODS

Materials. [^{3}H]ATP and unlabeled nucleotides were purchased from Schwarz/Mann, Orangeburg, N. Y., and P-L Biochemicals, Milwaukee, Wis., respectively. Oligoadenylate primers and poly(A) (6.2 S) were obtained from Miles Laboratories, Kankakee, Ill. Materials used in purification of poly(A) polymerase were prepared as previously described (9). Bovine lymphosarcoma, obtained through the courtesy of Dr. R. Marshak of the Veterinary School of the University of Pennsylvania, and calf thymus, obtained from a local abattoir, were stored at −90° until used.

ATP Analogs. Aza-ϵATP and DF-ϵATP were prepared as previously described (26). ϵATP was prepared according to the procedure of Secrist *et al.* (16). The structures of these ATP analogs are shown in Chart 1. In ϵATP, the N-6 amino group of ATP has been bridged with N-1, creating a fluorescent ring structure. Removal of the C-2 moiety produces DF-ϵATP, which can be further converted to aza-ϵATP by re-

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[3] The abbreviations used are: aza-ϵATP, 2-aza-1,N^6-etheno-adenosine triphosphate; poly(A), polyadenylate; DNA polymerase, deoxynucleoside triphosphate:DNA deoxynucleotidyltransferase (EC 2.7.7.7); RNA polymerase, nucleoside triphosphate:RNA nucleotidyltransferase (EC 2.7.7.6); poly(A) polymerase, ATP:polynucleotide adenyltransferase (EC 2.7.7.19); DF-ϵATP, 3-β-D-ribofuranosyl-4-amino-5-(imidazol-2-yl) imidazole triphosphate; ϵATP, 1,N^6-etheno-adenosine triphosphate; poly(aza-ϵA), a polymer of 2-aza-1,N^6-etheno-adenosine monophosphate; ara-ATP, 9-β-D-arabinofuranosyladenine-5'-triphosphate; $(A_n)_mA$, an oligomer containing n 3'-AMP residues and a 3'-terminal adenosine; aza-ϵAMP, 2-aza-1,N^6-etheno-adenosine monophosphate.

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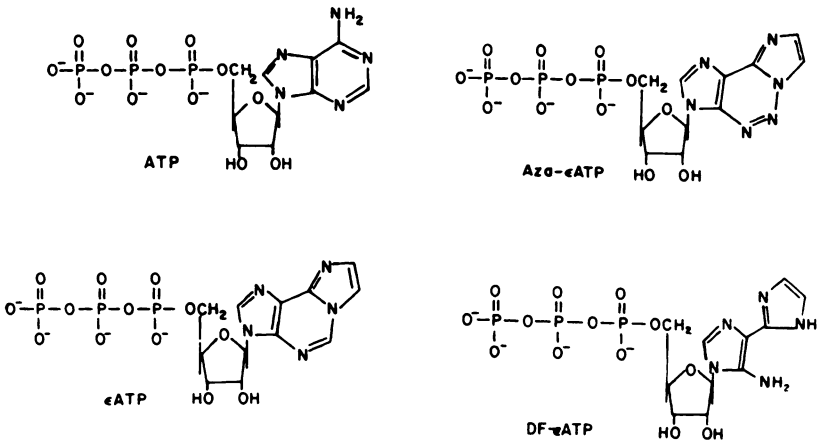

Chart 1. Structures of ATP and the analogs εATP, DF-εATP, and aza-εATP.

placing a nitrogen atom in the 2 position.

Poly(aza-εA), with an average chain length of 100 nucleotides, was synthesized from aza-εADP with the use of polynucleotide phosphorylase (21). For shorter polymers, poly(aza-εA) (0.1 μmole nucleotides) and poly(A) (0.5 μmole nucleotides) were treated with RNase A (4 μg/ml) in 0.015 M sodium chloride (pH 7.0) containing 0.0015 M sodium citrate overnight at 25°. Alkaline phosphatase (200 μg/ml) was also present in order to remove 3'-phosphate groups generated by the RNase. The products were acid precipitated and resuspended in 0.01 M Tris-Cl (pH 7). Polyacrylamide gel electrophoresis showed that the products were one-half or less the length of the starting material.

ara-ATP was synthesized as described by Furth and Cohen (3, 4).

Enzymes. DNA polymerase α was purified as previously described (6, 23). RNA polymerases II and III were purified from calf thymus as previously described (7, 8). (For RNA polymerase III, fractions with activity eluting from DEAE-Sephadex were combined and concentrated with ammonium sulfate.) Poly(A) polymerase was obtained from calf thymus and bovine lymphosarcoma as described previously (9). The hydroxyapatite fraction was used in the experiments reported. The specific activity of the enzymes were (units/mg protein): 8,400, bovine lymphosarcoma; 10,000, calf thymus.

Enzyme Assays. Poly(A) polymerase was assayed in a reaction mixture (125 or 250 μl) containing 100 mM Tris-HCl (pH 8.3), 2 mM $MnCl_2$, 40 mM $(NH_4)_2SO_4$, 4 mM 2-mercaptoethanol, 12 mM $(Ap)_3A$ or $(Ap)_5A$, 1 mM [^{3}H]ATP (500 to 1500 cpm/nmole), and enzyme. After 30 min at 37°, incorporation of labeled triphosphate into poly(A) was determined as described previously (9). One unit of poly(A) polymerase is defined as the amount of enzyme that converts 1 nmole of [^{3}H]ATP into acid-insoluble material in 60 min under standard reaction conditions. Incorporation of aza-εATP was measured in reaction mixtures (250 μl) containing 1 mM [^{3}H]ATP (500 cpm/nmole), 10 mM $(Ap)_5A$, 40 units of lymphosarcoma poly(A) polymerase and 0, 200, and 400 μM aza-εATP. After 60 min at 37° the reaction was terminated by heating at 100° for 1 min and 20-μl aliquots were removed for determination of polynucleotide synthesis by radioactivity. Unincorporated nucleoside triphosphate was removed by dialysis at 0° against 0.15 M NaCl containing 0.015 M sodium citrate (for 6 hr, 3 times) and then against 0.1 M potassium phosphate (pH 7.0) for 8 hr, 2 times. Aza-εATP incorporation was determined by fluorescence (21).

DNA polymerase and RNA polymerase were assayed as described previously (8, 23).

RESULTS

The effect of aza-εATP on mammalian nucleotide-polymerizing enzymes is shown in Table 1. DNA polymerase α is not inhibited by aza-εATP, and Form III RNA polymerase is only slightly inhibited, even at a concentration that is almost 4-fold higher than substrate. Form II RNA polymerase is inhibited by aza-εATP, but a 4-fold excess of aza-εATP inhibits only 32%. Poly(A) polymerase, in contrast, is almost entirely inhibited by a 3-fold excess of aza-εATP.

The effect of various concentrations of aza-εATP on bovine lymphosarcoma poly(A) polymerase, together with the effect of other ATP analogs, is shown in Chart 2 (*top*). The diphosphate analog ADP does not affect the reaction. εATP and DF-εATP are poor inhibitors, compared to aza-εATP, reducing incorporation of AMP by only 40% at twice the concentration of substrate. ara-ATP, a potent inhibitor of mammalian DNA polymerase α but not mammalian RNA polymerase (3, 4), has some inhibitory effect on poly(A) polymerase. The analogs of ATP that are the most effective inhibitors of poly(A) polymerase are dATP and aza-εATP. These analogs inhibit AMP incorporation approximately 50% at a concentration equal to that of ATP.

Inhibition of calf thymus poly(A) polymerase by analogs of ATP is similar to that observed with the enzyme of bovine lymphosarcoma (Chart 2, *bottom*). ADP does not inhibit the enzyme; aza-εATP and dATP are both effective inhibitors.

In order to determine the mode of inhibition by aza-εATP, the effect of a single level of the analog was measured at varying concentrations of ATP (Chart 3). Linear regression analysis indicates that aza-εATP inhibits poly(A) polymerase competitively. The K_i of aza-εATP, as determined from the intercepts in the plot of this experiment, is 260 μM, with a K_m for ATP of 580 μM. This suggests that aza-εATP has twice as much affinity for poly(A) polymerase as does ATP. However, K_m values have varied depending on how the assay is per-

Table 1

Inhibition of mammalian nucleotide-polymerizing enzymes by aza-ϵATP

Assays were performed as described in "Materials and Methods," except that the concentration of ATP (or dATP in the case of DNA polymerase α) was 160 μM. The concentration of aza-ϵATP was 600 μM, except that in the reaction with calf thymus poly(A) polymerase, the concentration of aza-ϵATP was 400 μM. One hundred % activity represents the activity observed in the absence of inhibitor.

Enzyme	Relative activity (%)	No. of determinations
Bovine lymphosarcoma DNA polymerase α	98 ± 19[a]	4
Calf thymus DNA polymerase α	115 ± 7	2
Calf thymus RNA polymerase III	80 ± 7	2
Calf thymus RNA polymerase II	68 ± 14	5
Calf thymus poly(A) polymerase	14 ± 4	4
Bovine lymphosarcoma poly(A) polymerase	15 ± 3	2

[a] Mean ± S.D.

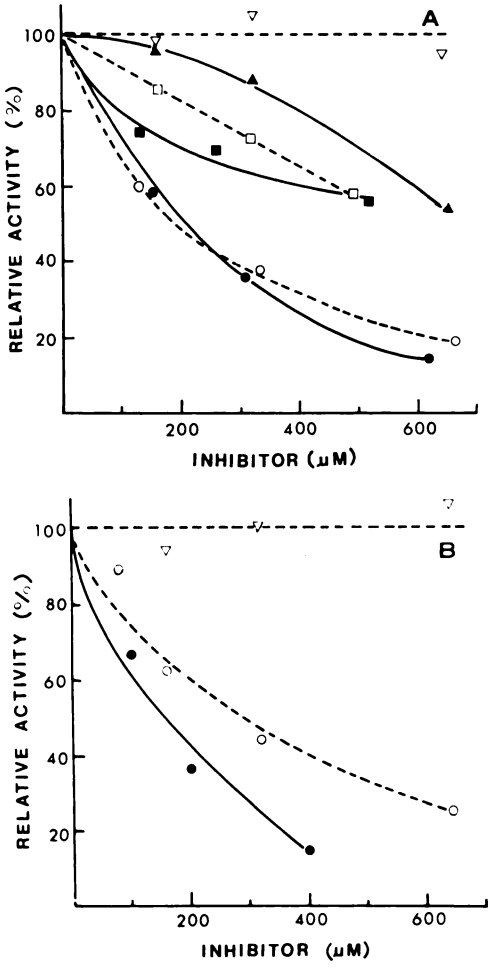

Chart 2. Inhibition of poly(A) polymerase by analogs of ATP. *A*, bovine lymphosarcoma; *B*, calf thymus. Standard assay conditions with $(Ap)_3A$ as primer, 200 μM [^{3}H]ATP, and 18 to 36 units of poly(A) polymerase. Inhibitor was present at the concentrations indicated. ●, aza-ϵATP; ○, dATP; ■, DF-ϵATP; □, ϵATP; ▲, ara-ATP; ▽, ADP.

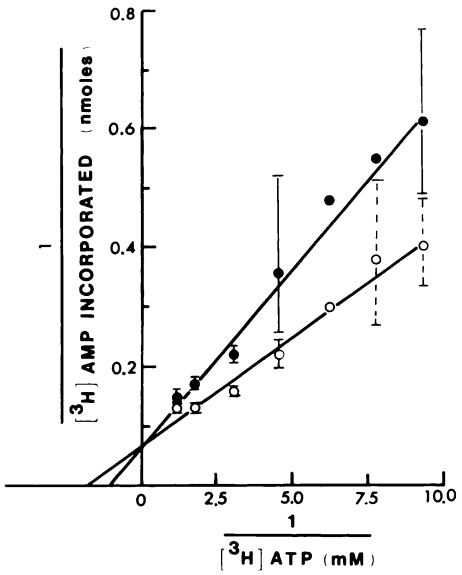

Chart 3. Inhibition of bovine lymphosarcoma poly(A) polymerase by aza-ϵATP. Standard reaction conditions were used, with $(Ap)_3A$ as primer, 160 μM aza-ϵATP, and 36 units of poly(A) polymrase. [^{3}H]ATP (1000 cpm/nmole) was varied from 100 to 850 μM. Duplicate aliquots (0.1 ml) were taken from each reaction mixture; results are reported as incorporation per aliquot. Each *point* represents an average of 1 to 3 determinations. The *slopes* and *intercepts* were determined by linear regression analysis; ○, without aza-ϵATP; ●, with aza-ϵATP.

formed, and the assay is nonlinear at short reaction times or low substrate concentrations. Although the experiments reported here were performed in the linear portion of the assay curve, the values of K_i and K_m should be regarded as tentative.

Competitive inhibition could result from one or more of several mechanisms: aza-ϵATP may (*a*) bind to the substrate site without being incorporated into the nascent poly(A) chain, (*b*) be incorporated very slowly, thus slowing the overall reaction, or (*c*) be incorporated into a poly(A) chain and terminate synthesis of that chain. In order to examine the latter 2 possibilities (which assume some incorporation of the inhibitor), poly(A) was synthesized in the presence of 2 concentrations of aza-ϵATP, as described in "Materials and Methods." Incorporation of 40 nmoles of [^{3}H]AMP was observed in the absence of aza-ϵATP; 30 and 25 nmoles were incorporated in the presence of 200 and 400 μM aza-ϵATP, respectively. Recovery of acid-precipitable radioactivity after processing was 23%; the concentration of incorporated AMP in the control sample was 10 nmoles/ml. No incorporation of aza-ϵAMP was observed by fluorescence in experimental samples (in which the inhibitor was present during the reaction) compared to samples in which an equal

amount of aza-ϵATP had been added after termination of the reaction. Since fluorescence can detect as little as 0.1 nmole aza-ϵ-adenosine per ml (21), less than 1 aza-ϵAMP was incorporated for every 100 AMP residues. Thus, aza-ϵATP inhibits competitively by Mechanism *a*; it binds to the substrate site without being incorporated.

The mode of inhibition by dATP was also tested by varying the concentration of ATP at a constant level of inhibitor. The data gave a similar pattern of competitive inhibition, with a K_i for dATP about one-half the level of the K_m for ATP.

Since dATP inhibits poly(A) polymerase, the inhibition of the enzyme by other naturally occurring nucleoside triphosphates was investigated (Table 2). The purine nucleotides GTP and dGTP are slightly more inhibitory than pyrimidine nucleotides. Deoxynucleotides are more potent inhibitors than the corresponding ribonucleotides; the difference in inhibition is most significant in the case of UTP and dTTP, where there is a difference in the ring structure as well as the sugar moiety. These data, together with the inhibition observed with dATP, suggest that purine nucleoside triphosphates may be more effective inhibitors than pyrimidine nucleoside triphosphates and that deoxynucleotides may be more effective than ribonucleotides. Only dATP is as effective an inhibitor as aza-ϵATP.

Previous work has shown that the primer site of poly(A) polymerase is much less specific than the substrate site (9). In view of the inhibitory properties of aza-ϵATP, the ability of an aza-ϵAMP polymer, poly(aza-ϵA), to serve as a primer for poly(A) polymerase of lymphosarcoma was tested (Chart 4) and compared with oligo(A) [$(Ap)_5A$] and poly(A) primers. All primers were at or near the saturating concentration. As reported previously (9), poly(A) is a relatively poor primer for poly(A) polymerase compared with oligo(A). Poly(aza-ϵA) is less effective than poly(A), but appears to support poly(A) synthesis at a rate slightly greater than that observed in the absence of any added primer. Similar results are obtained with poly(A) polymerase of calf thymus. Poly(aza-ϵA) and poly(A) were also subjected to RNase degradation, as described in "Materials and Methods." The shorter polymers demonstrated the same relative abilities to support poly(A) synthesis as the results shown in Chart 4. Thus both relatively short and long polymers of aza-ϵAMP are able to prime poly(A) synthesis, consistent with the less specific nature of the primer site compared to the substrate site.

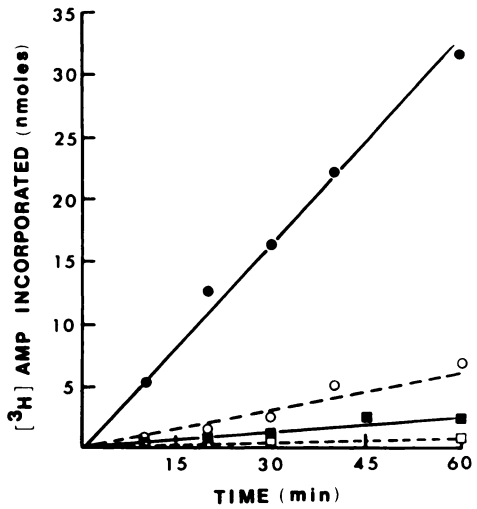

Chart 4. Poly(aza-ϵA) as primer for bovine lymphosarcoma poly(A) polymerase. Standard assay conditions, except for primer; [^{3}H]ATP (1 mM) at 8700 cpm/nmole and 36 units of poly(A) polymerase were used. Aliquots (40 μl) were taken from the reaction mixture (total volume 0.5 ml) at the times indicated. ●, $(Ap)_5A$ (10 μM); ○, poly(A) (8 μM); ■, poly(aza-ϵA) (8.6 μM); □, no primer.

Table 2
Inhibition of bovine lymphosarcoma poly(A) polymerase by ribo- and deoxynucleotides

Assays were performed using standard conditions with $(Ap)_3A$ as primer, 200 μM [^{3}H]ATP (1000 cpm/nmole), and 18 to 36 units of bovine lymphosarcoma poly(A) polymerase. Inhibiting ribo- or deoxynucleotides were present at a concentration of 400 μM. Values represent the mean ± S.D. of 4 to 6 determinations. One hundred % activity represents the activity observed in the absence of inhibitor.

Nucleoside triphosphate	Relative activity (%)	Deoxynucleoside triphosphate	Relative activity (%)
GTP	68 ± 11	dGTP	52 ± 13
UTP	92 ± 9	dTTP	59 ± 11
CTP	93 ± 16	dCTP	71 ± 14
		dATP	30[a]

[a] Value obtained from the experiment reported in Chart 2.

DISCUSSION

This study has shown that mammalian poly(A) polymerase is significantly inhibited by a synthetic substrate analog, aza-ϵATP, and by a natural analog, dATP. Other synthetic and natural nucleoside triphosphates also inhibit to varying degrees. ATP analogs that possess the etheno group but not the 2-aza substitution are poor inhibitors of poly(A) polymerase. This is consistent with previous observations indicating that aza-ϵADP is a better substrate than ϵADP for polynucleotide phosphorylase (21). As suggested by Tsou and Yip (21), the nitrogen in position 2 may take the place of the N^1 position in forming one of the bonds at the active site.

The natural nucleotide that is the most effective inhibitor is dATP, the deoxy analog of the natural substrate. Other purine nucleotides are as a group more effective than pyrimidine nucleotides; deoxynucleoside triphosphates are in general more inhibitory than ribonucleoside triphosphates. It is not surprising that purine nucleoside triphosphates (ribo- or deoxy-) are more effective than the pyrimidine nucleotides, since the normal substrate, ATP, bears a closer structural resemblance to other purine nucleotides than to the pyrimidine nucleotides. It is interesting that deoxynucleoside triphosphates inhibit more than their ribonucleotide counterparts. Poly(A) polymerase appears to discriminate between ribose and arabinose moieties, since ara-ATP is a relatively ineffective inhibitor. In this regard, it is somewhat surprising that the enzyme cannot discriminate between the deoxyribose and ribose moiety, but instead is more susceptible to inhibition by nucleotides containing deoxyribose than by nucleotides containing the ribose group of the natural substrate.

The ability of deoxynucleotides to inhibit poly(A) polymerase may have biological significance. Deoxynucleotide concentrations in mammalian cells are much higher in the S phase than at other times in the cell cycle (18), and deoxynucleotides are located predominantly within the nucleus during the S phase (17). Histone mRNA's, which are synthesized during S, lack poly(A) (1); it is conceivable that the failure of polyadenylation of histone mRNA's may be related to the inhibition of poly(A) polymerase by the high nuclear concentrations of deoxynucleotides during S phase.

Both dATP and aza-ϵATP appear to inhibit poly(A) polymerase from a neoplastic tissue (lymphosarcoma) to the same extent as the enzyme from a related normal tissue (thymus). Since the amounts of the enzyme are also similar in the 2 tissues (9), it would not appear likely that aza-ϵATP would be useful in therapy of this tumor. Furthermore, since ATP is a substrate in many cellular reactions, the aza-ϵ series of ATP analogs may not possess enough specificity for tumor cell metabolism to be useful as chemotherapeutic agents; in the case of the triphosphate analog aza-ϵATP, its ability to be transported into cells would also require investigation. However, in view of the inhibitory effects of aza-ϵ adenosine on tumor cell lines *in vitro* (22), the possible chemotherapeutic effectiveness of aza-ϵ compounds merits further investigation. For example, the effects of these compounds on other cellular processes, such as glycolysis and active transport, would be of interest.

The inhibition of poly(A) polymerase by aza-ϵATP is fairly specific; DNA polymerase α is unaffected, and RNA polymerases II and III are inhibited one-third or less by a several-fold excess of the compound. Although cordycepin is thought to inhibit poly(A) polymerase *in vivo* after phosphorylation (2, 11), recent work suggests that cordycepin triphosphate *in vitro* is a potent inhibitor of RNA polymerase II as well as poly(A) polymerase (10). Consistent with this, Milcarek *et al.* (12) have shown that mRNA lacking poly(A) is significantly inhibited by cordycepin. The preferential inhibition of mRNA produced by cordycepin *in vivo* may thus be due to a combination of effects. In this regard, aza-ϵATP may prove useful in studies requiring a selective inhibition of poly(A) polymerase *in vivo*. Such studies would help to elucidate poly(A) metabolism in both normal and neoplastic cells.

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