Effect of 3′-Amino-3′-deoxyadenosine on Nucleic Acid Synthesis in Ehrlich Ascites Tumor Cells

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SUMMARY

3′-Amino-3′-deoxyadenosine strongly inhibits nucleic acid labeling in Ehrlich ascites tumor cells. The compound is shown to be converted to corresponding 5′-phosphates in the cells. 3′-Amino-3′-deoxy-ATP blocks RNA formation as catalyzed by the aggregate enzyme and by isolated nuclei. It is suggested that growing RNA chains incorporate the 3′-amino-3′-deoxyadenosine 5′-phosphate into their free 3′-OH end groups, and are thereafter unable to support further synthesis.

INTRODUCTION

The antitumor agent 3′-amino-3′-deoxyadenosine (structure shown in Fig. 1) is

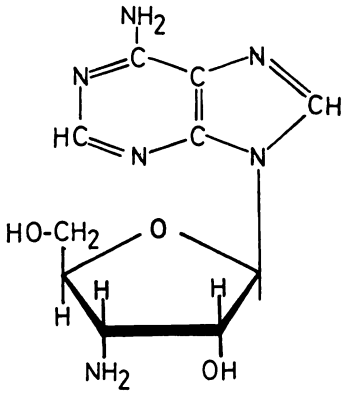

FIG. 1. *Structural formula of 3′-amino-3′-deoxyadenosine*

active against a wide variety of ascites tumors in mice (1, 2). It is isolated from culture filtrates of the turf fungus *Helminthosporium* sp. no. 215 (3). Because of its structural similarity to 3′-deoxyadenosine, an antitumor agent derived from the mold *Cordyceps militaris* (4), a comparison of their effects on RNA and DNA synthesis has been made. This report proposes to show that the inhibitory effect of 3′-amino-3′-deoxyadenosine on RNA synthesis in Ehrlich ascites tumor cells may be ascribed to its phosphorylation to the corresponding 5′-triphosphate. This compound can be isolated from tumor cells and is shown to be a potent inhibitor of DNA-dependent RNA polymerization in both isolated nuclei and a homologous ascites cell RNA polymerase preparation.

A preliminary report of these results has been published elsewhere (5).

MATERIALS AND METHODS

3′-Amino-3′-deoxyadenosine derived from *Helminthosporium* was kindly supplied by Dr. H. A. Lechevalier, Rutgers University, New Brunswick, New Jersey.

ATP, GTP, UTP, and CTP were obtained from the Sigma Chemical Co., St. Louis, Missouri; ATP-8-^{14}C and GTP-^{3}H were obtained from Schwarz Bio-Research Inc. Orangeburg, New York; adenine-8-^{14}C was obtained from the Radiochemical Centre, Amersham, England.

Ehrlich ascites tumor cells were used 6–7 days after transplantation in strain NMR mice.

The mixtures for determination of the incorporation of labeled precursors into RNA, DNA, or ribonucleotides were preincubated for 15 minutes with or without 3′-amino-3′-deoxyadenosine before addition

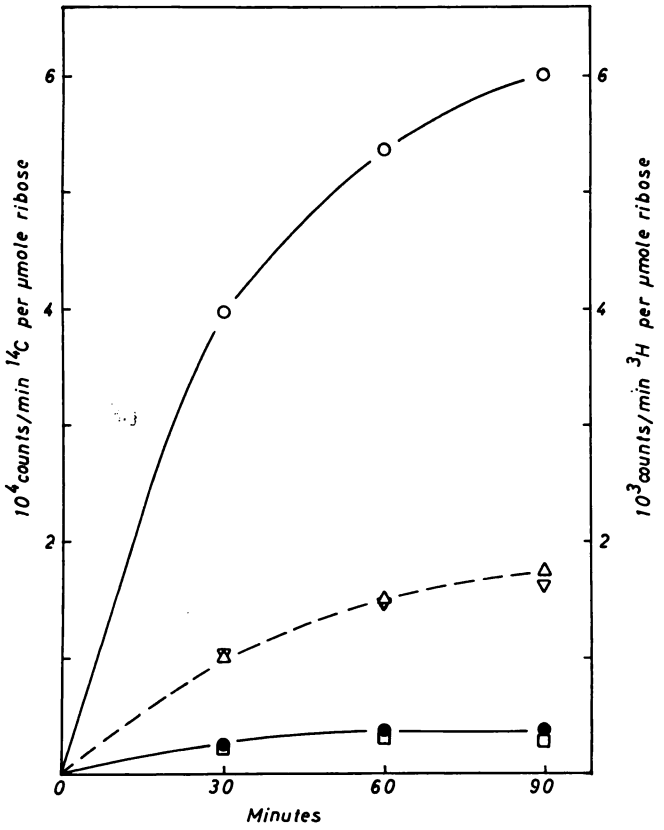

FIG. 2a. *Effect of 3'-amino-3'-deoxyadenosine on the incorporation of adenine-^{14}C and uridine-^3H into RNA of Ehrlich ascites tumor cells in vitro*

Each flask contained per milliliter: Ehrlich ascites tumor cells 80 mg (wet weight); ascites fluid, 500 μl; Robinson's medium (26) containing glucose (5.6×10^{-3} M), 300 μl; H_2O, 150 μl; folic acid, 20 mμmoles, and 3'-amino-3'-deoxyadenosine as indicated. After incubation for 15 min uridine-^3H, 16.5 μC, (1 μmole carrier uridine) and adenine-^{14}C, 1.25 μC (28.3 μC/μmole) were added. Separation of ^3H and ^{14}C activities was by the discriminator-ratio method (27) in a Packard Tri-Carb spectrometer.

Adenine-^{14}C incorporation with 3'-amino-3'-deoxyadenosine added (per ml): ○——○, none; △——△, 0.3 μmole; ●——● 2.2 μmoles. Uridine-^3H incorporation with 3'-amino-3'-deoxyadenosine added (per ml): ○——○, none; ▽——▽ 0.3μmole; □——□ 2.2 μmoles.

of the labeled compound (details in legend, Fig. 2). Incubation was performed at 37° with shaking. Samples were treated with 0.5 *N* perchloric acid at 0°, and the specific activities of RNA and DNA present in the acid-insoluble fraction were determined by standard means (6–8). The size and specific activity of the pool of acid-soluble ribonucleotides were determined after isolation by paper chromatography as previously described (9). The amount of ribonucleotides in each sample was determined by cutting out the appropriate ultraviolet absorbing spots, eluting with water, and determining the ribose content by orcinol assay (7) before and after treatment with Norit®. Radioactivity was measured in a liquid scintillation spectrometer.

Phosphorylation products of 3'-amino-3'-deoxyadenosine were isolated from the acid-soluble fraction of cells by chromatography on a Dowex-1 anion exchange column according to a modification of the method previously described for 3'-deoxyadenosine (10). Three prominent peaks, later identifiable as containing the 3'-amino-3'-deoxy-

nucleotides, were eluted (see Fig. 4). These were precipitated as barium salts in the following manner: 1/100 volume of 1 M glycine, pH 9.2, was added to each fraction and the pH was adjusted to 8.5 with KOH; then 1 volume of cold ethanol containing 1/100 volume of saturated $BaBr_2$ was added. After 18 hours the precipitate was dissolved in water at 0°, and treated with Dowex 50 (NH_4^+ form) (11). After stirring, the supernatant solution was removed and the resin was washed twice with water. The combined supernatant fractions were assayed for acid-labile phosphate compounds by the method of Fiske and SubbaRow after hydrolysis at 100° in 1 N H_2SO_4 for 10 min (12). Reduction of periodate was determined by the spectrophotometric method (13). Ribose was assayed by reaction with orcinol (7); deoxyribose by reaction with diphenylamine (8). Complexing with borate was determined by chromatography (9).

Adenosine deaminase (adenosine aminohydrolase, EC 3.5.4.4) was prepared according to Coddington (14); apyrase (ATP diphosphohydrolase, EC 3.6.1.5) was prepared by fractionation of potato extract (15); and snake venom 5′-nucleotidase (5′-ribonucleotide phosphohydrolase, EC 3.1.3.5) was obtained by extraction of freeze-dried venom (Ross Allen's Reptile Farm, Florida) with water (16).

Cell-free DNA-dependent RNA polymerase (nucleosidetriphosphate:RNA nucleotidyltransferase, EC 2.7.7.6) was prepared from Ehrlich ascites tumor cells according to the method of Goldberg (17).

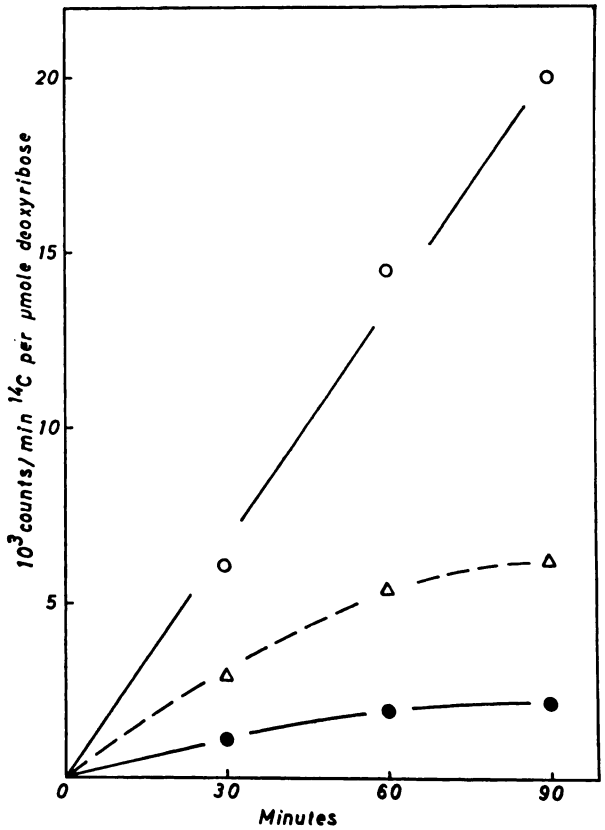

FIG. 2b. *Effect of 3′-amino-3′-deoxyadenosine on the incorporation of adenine-^{14}C into DNA of Ehrlich ascites tumor cells in vitro*

Conditions of incubation were the same as in (a). 3′-Amino-3′-deoxyadenosine added (per ml): ○——○, none; △——△, 0.3 μmole; ●——● 2.2 μmoles.

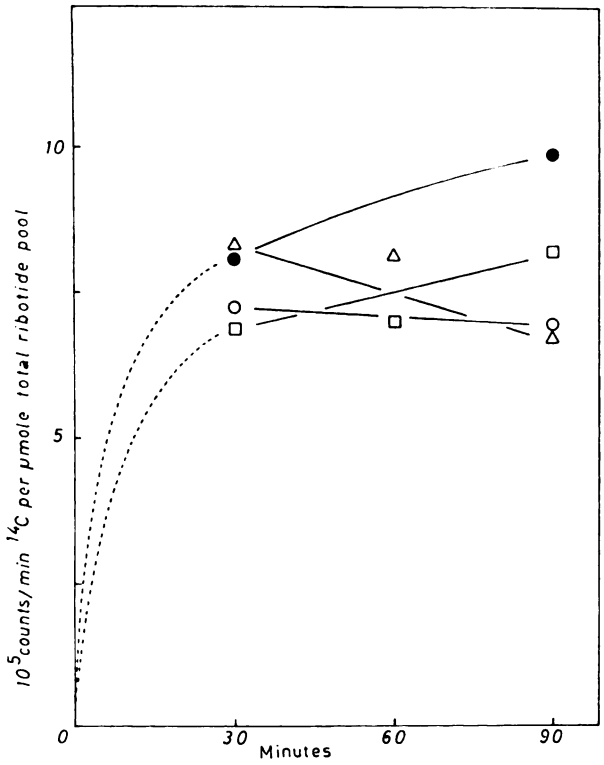

FIG. 3a. *Effect of 3'-amino-3'-deoxyadenosine on the specific activity of the acid-soluble ribonucleotide pool of Ehrlich ascites tumor cells labeled with adenine-^{14}C*

Experimental conditions as described for Fig. 2. Preparation of the acid-soluble ribonucleotide pool as described under Materials and Methods. 3'-Amino-3'-deoxyadenosine added (per ml): ○—○, none; △—△, 0.3 μmole; □—□ 1.2 μmoles; ●—● 2.2 μmoles.

RNA-synthesis in the presence of UTP, CTP, GTP, and ATP-^3H was assayed by the filter paper disk method of Bollum (18).

RNA synthesis in isolated nuclei was determined by a modification of the method of Takahashi *et al.* (19). Ehrlich cells were swollen in hypotonic sucrose, frozen 10 min at −42°, thawed in cold tapwater for 10 min, frozen again, thawed, and made isotonic with hypertonic sucrose. This gave a preparation of 96% clean nuclei when examined by phase contrast microscopy after washing in 2% citric acid. The incubation mixture of nuclei for the measurement of RNA synthesis was the same as described by Takahashi *et al.* (19), using GTP-^3H as tracer. Cell-free DNA polymerase (deoxynucleosidetriphosphate : DNA deoxynucleotidyltransferase, EC 2.7.7.7) was prepared from a high-speed supernatant fraction of Ehrlich cells according to the method of Keir and Shepherd (20). The enzymatic synthesis of acid-insoluble material in the presence of 2'-dATP, 2'-dCTP, 2'-dGTP, and TTP-^3H was assayed.

RESULTS

The effect of two different concentrations of 3'-amino-3'-deoxyadenosine on the incorporation of adenine-^{14}C and uridine-^3H into DNA and RNA was studied. The rate of incorporation of both compounds into RNA was inhibited 70–75% in the presence of 0.3 μmole of the analog per milliliter of cell suspension, and about 95% in the presence of 2.2 μmoles/ml (Fig. 2a).

The incorporation of adenine-^{14}C into DNA was inhibited to a slightly lesser

extent than into RNA, as shown in Fig. 2b. At a concentration of 0.3 μmole of 3′-amino-3′-deoxyadenosine per milliliter of cell suspension, there was 65% inhibition, and at 2.2 μmoles/ml there was 88% inhibition.

Such an inhibition of incorporation of labeling into the nucleic acids may be ascribed to any of the following effects. The analog may inhibit one or more of the steps leading to the incorporation of the labeled compounds into the corresponding immediate precursors of the nucleic acid; it may prevent the accumulation of a sufficient concentration of other immediate precursors required for nucleic acid synthesis; or it may inhibit the nucleic acid polymerase reaction.

Concerning RNA synthesis, the first of the above possibilities is probably excluded by the fact that there is no decrease in specific activity of the acid-soluble ribonucleotide pool at either low or high concentration of the analog (Fig. 3a). The second possibility is probably likewise excluded, at least as far as the low concentration of analog is concerned, by the fact that there is no decrease in size of the acid-soluble ribonucleotide pool (Fig. 3b). However, the composition of the pool was not analyzed. With increasing concentration of analog there is a progressive diminution in pool size, and this may be an additional contributing factor to the greater inhibition of RNA synthesis with higher concentrations of analog. It seems likely, therefore, that the inhibition of RNA synthesis at the lower concentration of analog (0.3 μmole/ml) is due to a specific inhibition of the DNA-dependent RNA polymerase reaction.

Previous experiments have indicated that

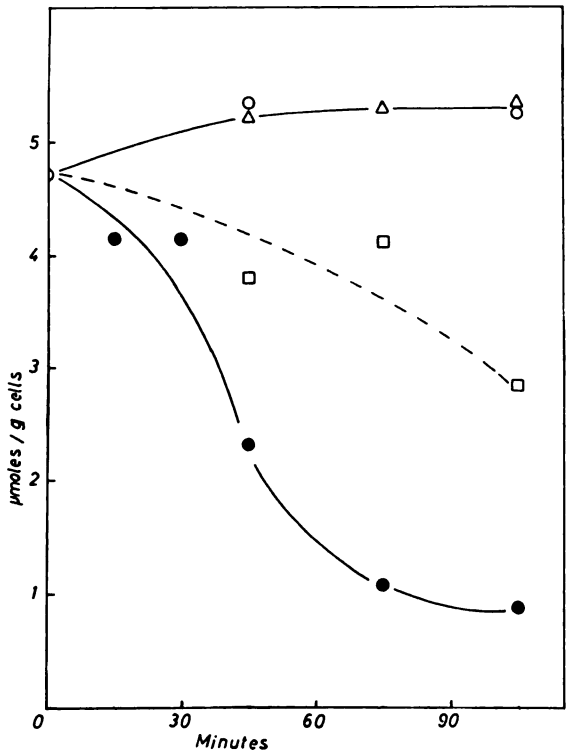

FIG. 3b. *Effect of 3′-amino-3′-deoxyadenosine on the size of the acid-soluble ribonucleotide pool of Ehrlich ascites tumor cells*

Experimental conditions as described for Figs. 2 and 3a. 3′-Amino-3′-deoxyadenosine added (per ml): ○——○, none; △——△, 0.3 μmole; □——□, 1.2 μmoles; ●——●, 2.2 μmoles.

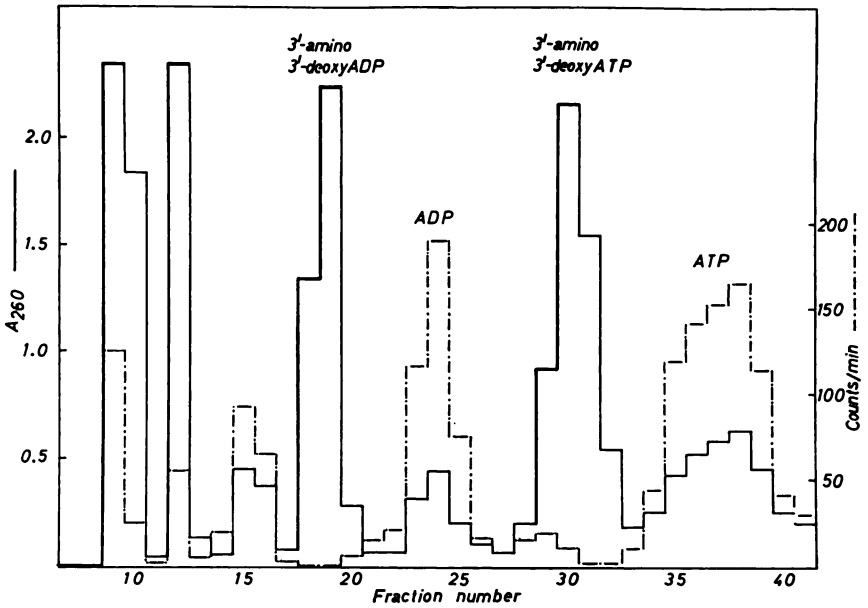

FIG. 4. *Dowex 1 chromatography of nucleotides from cells incubated with 3'-amino 3'-deoxyadenosine*

The incubation medium contained per ml: Ehrlich ascites tumor cells, 80 mg; ascites fluid, 500 μl; Robinson's medium containing glucose (5.6×10^{-3} M) 450 μl; folic acid 20 mμmoles; and ^{14}C-adenine (2.0 μC; 28.3 μC/μmole). Total volume 30 ml. After incubation for 45 min at 37°, 2.0 μmoles of 3'-amino-3'-deoxyadenosine was added per milliliter. After incubation for a further 150 min, the cells were collected by centrifugation and treated with 5 ml of cold 0.5 N $HClO_4$. The acid-soluble fraction was neutralized with 0.5 N KOH and passed through a Dowex 1 formate column (20×1.0 cm). The column was eluted by a linear concentration gradient from 0 to 2 M ammonium formate pH 4.5.

3'-amino-3'-deoxyadenosine, in the presence of ATP, myokinase, and adenosine kinase is converted to the corresponding 5'-triphosphate (21). Cells incubated with this nucleoside were therefore analyzed for their content of nucleotides. The adenosine phosphates were labeled by preincubation with adenine-^{14}C before the addition of 3'-amino-3'-deoxyadenosine. The acid-soluble fraction of these cells was chromatographed on an anion exchange resin and the fractions collected were analyzed. Three major ultraviolet absorbing peaks almost or completely devoid of radioactivity appeared in the chromatogram (Fig. 4). These had spectral characteristics as expected for adenine compounds (A_{280}/A_{260} 0.17–0.18 at pH 4.5). The slowest eluting of these peaks (fractions 29–32), migrated between two radioactive peaks which, according to their chromatographic behavior, could be identified as ADP and ATP, respectively. The ultraviolet absorbing material in this unlabeled peak was collected as a barium salt and converted to the ammonium salt as described in Materials and Methods. This compound had the following properties. On paper chromatography in ammonium acetate–ethanol solvent it migrated like ATP and 2'-deATP, whereas in borate–ammonium acetate–ethanol solvent it migrated like 2'-deATP with a greater R_f value than ATP, indicating that there was no complexing with borate. When adenosine deaminase was added to a buffered aqueous solution of the compound at pH 8.5 there was no deamination as measurable by a decrease in absorbancy at 265 mμ. Only after the addition of both apyrase and snake venom 5'-nucleotidase could deamination occur, 3'-amino-3'-deoxyadenosine being a known

substrate for adenosine deaminase (14). Assuming that the compound contained adenine with a molar extinction coefficient of 15.7 M^{-1} cm^{-1} at pH 7.0, it was furthermore found to contain 1.96 moles of acid-labile phosphate and to consume 0.95 mole of periodate per mole of adenine. The compound failed to give a positive reaction for either ribose (7) or 2′-deoxyribose (8). These findings are all consistent with the assumption that the isolated compound was 3′-amino-3′-deoxyadenosine triphosphate.

When the 3′-amino-3′-deoxyadenosine triphosphate was added to a homologous cell-free DNA-dependent RNA polymerase system there was a profound inhibition of incorporation of ATP-^{14}C into RNA (Fig. 5). The kinetics showed a complete blockade of the reaction even when the ratio between the concentration of ATP and 3′-amino-3′-deoxy-ATP was 50:1. Before this blockade became complete, however, there appeared to be an induction period of continuing RNA synthesis that varied directly with the molar ratio of ATP to 3′-amino-3′-deoxy-ATP. Similar kinetics of inhibition were seen with 3′-deoxy-ATP, although the degree of inhibition was slightly less.

Experiments with isolated cell nuclei showed a similar pattern of inhibition of incorporation of nucleoside triphosphates

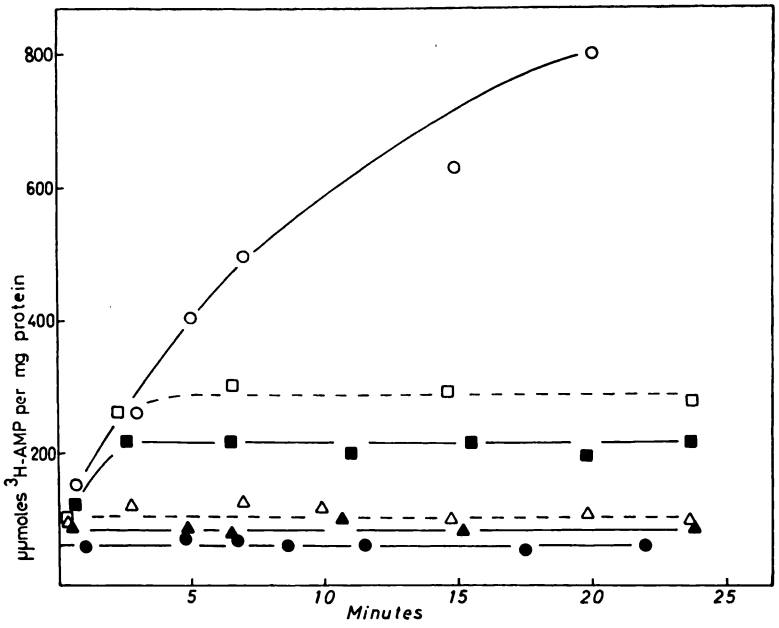

FIG. 5. *Effect of 3′-amino-3′-deoxy-ATP and 3′-deoxy-ATP on cell free DNA-dependent RNA polymerization*

Each reaction mixture contained in a total volume of 630 μl: 50 μmoles of Tris-HCl buffer (pH 8.0), 400 mμmoles of GTP, 400 mμmoles of CTP, 400 mμmoles of UTP, 200 mμmoles of ^{3}H-ATP (specific activity 3.1 × 10^{3} cpm/μmole), 1.9 μmoles of $MgCl_2$, 12.5 μmole of NaF, 50 μl of $(NH_4)_2SO_4$ (saturated at 0°), 0.4 μmole of phosphoenolpyruvate, 10 μg of pyruvate kinase, 10 μg of myokinase, and nucleotidyltransferase containing 1.34 mg protein determined by the method of Lowry (28). At the indicated times, 70-μl aliquots were pipetted onto filter paper disks (18), which were then immersed in ice cold 0.5 N perchloric acid and washed 6 times at 0° with 0.5 N perchloric acid (20 ml per disk). The disks were finally washed once in 96% ethanol containing 2% sodium acetate, once in 96% ethanol, dried, and counted in 10 ml of toluene scintillation liquid.

3′-Amino-3′-deoxy-ATP added: ○——○, none; ■——■, 4 mμmoles (ratio 3′-amino-3′-deoxy-ATP:ATP 1:50); ▲——▲, 40 mμmoles (1:5); ●——● 200 mμmoles (1:1). 3′-Deoxy-ATP added: □——□, 4 mμmoles (ratio 3′-deoxy-ATP:ATP 1:50; △——△, mμmoles (1:5).

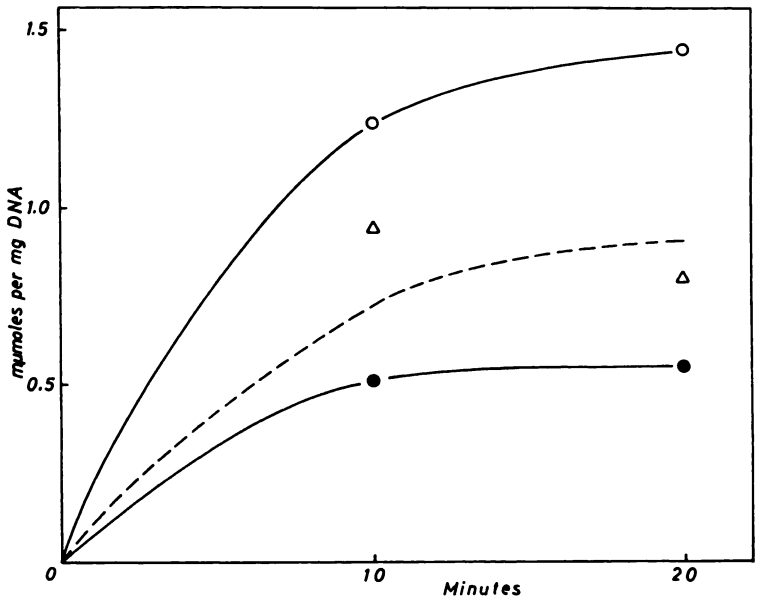

FIG. 6. *Effect of 3'-amino-3'-deoxy-ATP on the incorporation of ^{3}H-GMP into RNA of isolated Ehrlich ascites cell nuclei*

Each reaction mixture contained in a total volume of 2100 μl: 0.5 mmole of sucrose, 0.02 mg of dextran (MW 200,000–300,000), 0.06 mmole of potassium phosphate buffer (pH 8.0), 1.0 μmole of ATP, 0.5 μmole of CTP, 0.5 μmole of CTP, 0.5 μmole of ^{3}H-GTP (specific activity 1.5×10^6 cpm/μmole), 10 μmoles of $MgCl_2$, 5 μmoles of phosphoenolpyruvate, 300 μg of pyruvate kinase, and approximately 100 mg of isolated Ehrlich ascites cell nuclei. At the indicated times, 1.0-ml aliquots were withdrawn and added to 2.5 volumes 0.25 M sucrose at 0°, centrifuged, and washed twice in 10 volumes of cold 2% citric acid. To the washed nuclei was added 0.5 ml of ice cold 0.5 N perchloric acid, and extraction of RNA and DNA was performed by standard means (9). Uptake of ^{3}H-GTP was expressed as millimicromoles per millirgam of DNA in order to compensate for any slight variations in the number of nuclei per incubation mixture.

3'-Amino-3'-deoxy-ATP added: ○——○, none; △——△, 0.069 μmole (ratio 3'-amino-3'-deoxy-ATP : ATP 1:15); ●——●, 0.23 μmole (1:4).

into RNA (Fig. 6). However, this was not as profound on a mole per mole basis, and kinetic studies were rendered difficult by the fact that the time curve of the control nuclei bent off after incubation for 20 min.

An attempt was made to explain the inhibition of DNA labeling by 3'-amino-3'-deoxyadenosine. The effect of 3'-amino-3'-deoxy-ATP on the incorporation of TTP-^{3}H into DNA in the presence of 2'-dATP, 2'-dCTP, and 2'-dGTP was studied using a high-speed supernatant fraction of Ehrlich ascites tumor cells (20). At molar ratios of 3'-amino-3'-deoxy-ATP to ATP as high as 1:1 no inhibition could be demonstrated.

CONCLUSION

3'-Amino-3'-deoxyadenosine, like its structural analog 3'-deoxyadenosine, is a powerful inhibitor of the incorporation of adenine-^{14}C and uridine-^{3}H into both RNA and DNA in Ehrlich ascites tumor cells. A similar degree of inhibition of incorporation of hypoxanthine-^{14}C into total cell nucleic acids has been demonstrated by Shigeura *et al.* (21). Both 3'-amino-3'-deoxyadenosine and 3'-deoxyadenosine are capable of exerting considerable inhibition at concentrations which do not diminish the size of the ribonucleotide pool. Both agents are known to be substrates for partially purified adenosine kinase: K_m 3'-amino 3'-

deoxyadenosine = 6.1×10^{-4} M; K_m 3′-deoxyadenosine = 4.6×10^{-4} M (K_m adenosine = 1.6×10^{-6} M) (22). Incubation of 3′-amino-3′-deoxyadenosine with Ehrlich ascites cells results in the intracellular accumulation of three ultraviolet-absorbing compounds. One of these was identified as the corresponding 5′-triphosphate. 3′-Amino-3′-deoxyadenosine triphosphate has a pronounced inhibitory effect on homologous cell free DNA-dependent RNA formation, and on RNA synthesis in isolated nuclei. The degree of inhibition is approximately the same as that seen with equimolar concentrations of 3′-deoxy-ATP. This similarity in degree of inhibition is to some extent in disagreement with the results of Shigeura *et al.* (21), who found 3′-deoxy-ATP to be a more effective inhibitor using a *Micrococcus lysodeikticus* RNA polymerase system. The kinetics of inhibition are of the type expected to result from the irreversible incorporation of a nucleoside monophosphate lacking a 3′-OH group into the 3′ end of a polyribotide chain growing in the 5′ to 3′ direction (Fig. 7).

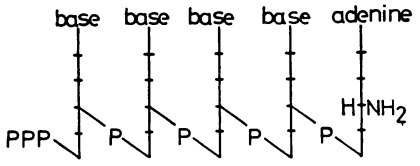

FIG. 7. *Schematic diagram showing a polyribonucleotide chain of the type proposed to be formed in the presence of 3′-amino-3′-deoxyadenosinetriphosphate, i.e., a chain containing a 3′-NH_2 end group instead of a 3′-OH end group*

No definite conclusions can be drawn about the inhibitory effect of 3′-amino-3′-deoxyadenosine on the incorporation of labeling into DNA, as the size and radioactivity of the pool of 2′-deoxyribotides were not determined. Since the activity of a cell-free DNA polymerase preparation was uninhibited by 3′-amino-3′deoxy-ATP it is likely that the effect seen on DNA synthesis in whole cells may be ascribed to inhibition of the ribonucleotide reductase enzyme. This enzyme is known to be under the allosteric influence of a number of nucleotides (23, 24).

One interesting difference in the effects of these two adenosine analogs is noted: at low or high concentrations 3′-amino-3′-deoxyadenosine in contrast to 3′-deoxyadenosine (25), does not cause any pronounced decrease in the specific activity of the acid-soluble ribonucleotide pool labeled with adenine-^{14}C. This difference may be explained either by a lack of inhibitory effect of 3′-amino-3′-deoxy-ATP on the formation of phosphoribosylpyrophosphate in contrast to what has been found for 3′-deoxy-ATP (25), or by a sufficiently slow accumulation of 3′-amino-3′-deoxy-ATP to allow the formation of a pool of phosphoribosylpyrophosphate from glucose.

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