Effect of Cordycepin on Nucleic Acid Metabolism in L5178Y Cells and on Nucleic Acid-synthesizing Enzyme Systems

Werner E. G. Müller, Gerhard Seibert, Rudolf Beyer, Hans J. Breter, Armin Maidhof, and Rudolf K. Zahn

SUMMARY

Cordycepin [3'-deoxyadenosine (3'-dAdo)] is an adenosine analog that interferes with nucleic acid synthesis both in vivo and in vitro. Our in vivo studies were performed with L5178Y cells. 3'-dAdo strongly inhibited cell proliferation (concentration that induces 50% inhibition of cell proliferation, 0.27 μM); 3'-dAdo-treated cells did not show unbalanced growth. The inhibitory potency of 3'-dAdo could be abolished to some extent by coinhibition with adenosine, but not with 2'-deoxyadenosine. In precursor studies, 3'-dAdo strongly reduced protein synthesis and to a lesser extent total RNA synthesis. The reduction of protein synthesis was most probably the result of the inhibition of mRNA synthesis, since in the presence of 3'-dAdo the number of polysomes decreased. In an intact cell system, [3H]-3'-dAdo was incorporated into RNA but not into DNA. Incorporation of [3H]-3'-dAdo was found in the polyadenylate (poly(A)) stretch of poly(A)-containing RNA and mainly in the 10 S and 55 S species of poly(A)-free RNA.

Cordycepin triphosphate (3'-dATP) had no influence on the activity of DNA-dependent DNA polymerase α and β from L5178Y cells. The incorporation rate of adenosine triphosphate into RNA by DNA-dependent RNA polymerases I, II, and III from mouse liver was moderately inhibited by 3'-dATP. The strongest inhibitory effect of 3'-dATP was observed in the enzyme systems containing nuclear poly(A) polymerase (from oviduct) or cytoplasmic terminal riboadenylate transferase (from calf thymus). The inhibition type observed in the enzyme systems containing nuclear poly(A) polymerase and terminal riboadenylate transferase, the enzyme activity was also inhibited competitively with respect to the oligo(pA)10 initiator. 3'-dATP was used as substrate by poly(A) polymerase; incorporated 3'-deoxyadenosine 5'-monophosphate acted as chain terminator.

INTRODUCTION

3'-dAdo² is an antibiotic isolated from culture filtrates of Cordyceps militaris and Aspergillus nidulans (for review, see Ref. 62). 3'-dAdo has been found to be a potent inhibitor both of cell proliferation of bacteria and eukaryotic cells (62) and of virus growth (55). Growth of tumor cells (Ehrlich ascites and human epidermoid carcinoma 1 cells) in culture and in vivo is inhibited by 3'-dAdo by cytostasis, rather than by cytotoxicity (26, 54). There is already some information on the mode of action of this cytostatic agent.

In an intact cell system, 3'-dAdo is phosphorylated to 3'-dAMP, 3'-dADP, and 3'-dATP (29). The phosphorylated forms of the antibiotic are involved in the inhibition of several enzyme reactions; 3'-dAMP suppresses phosphoribosyl-pyrophosphate amidotransferase, and 3'-dATP influences the synthesis of 5-phosphoribosyl 1-pyrophosphate from ribose 5-phosphate and ATP, as well as that of DNA and RNA polymerases (for review, see Ref. 62). Craig (13) concluded that 3'-dAdo inhibits the production of rRNA and mRNA, as well as protein synthesis, at the level of initiation. Recent reports indicate that 3'-dAdo is not an unspecific inhibitor of nucleic acid synthesis, but rather a highly specific inhibitor of those RNA’s that contain poly(A) (for review, see Ref. 8). Consequently, 3'-dAdo gains some importance for cancer chemotherapy, since thus far no antitumor drug that interferes with mRNA maturation is available.

It was the aim of the present study to discover that site in a mouse tumor cell system (L5178Y cells) that is most sensitive to inhibition by 3'-dAdo. The studies were performed first with 3'-dAdo to determine its effect on an intact cell system and then with 3'-dATP to elucidate the influence of this compound in a series of DNA and RNA polymerases that have been isolated from eukaryotic systems.

MATERIALS AND METHODS

Compounds. The following materials were obtained: 3'-dAdo, Sigma Chemical Co., St. Louis, Mo.; poly[l-14C]uridine (specific activity, 1.1 μCi/μmole phosphorus) and 23 S + 16 S + 5 S + 4 S rRNA, Miles-Pentex Laboratories, Kankakee, Ill.; [methyl-3H]dThd (specific activity, 19 Ci/m mole), [G-3H]uridine (specific activity, 6.3 Ci/m mole), L-[4,5-3H]lysine (specific activity, 6.6 Ci/m mole), [3H]ATP (specific activity, 11 Ci/m mole), and [3-3H]dTd (specific activity, 9.5 Ci/m mole) aquasol, New England Nuclear, Boston, Mass.; poly(U)-Sephadex G-25, from Deutsche Pharmacia, Frankfurt, Germany; Whatman GF/C filters and Whatman

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1 We gratefully acknowledge loans from the Academy of Science and Letters, Mainz, Germany, and from the Fonds der chemischen Industrie.

2 The abbreviations used are: 3'-dAdo, 3'-deoxyadenosine (cordycepin); 3'-dAMP, 3'-deoxyadenosine monophosphate (cordycepin monophosphate); 3'-dATP, 3'-deoxyadenosine triphosphate (cordycepin triphosphate); poly(A), polyadenylate; poly(U), polyuridylicate; tThd, deoxythymidine; ED₅₀ concentration, concentration that inhibits cell proliferation by 50%; oligo(A), oligoadenylate.
DE 81 chromatography paper, from Hornuth and Vetter, Heidelberg, Germany; RNase A (pancreas), alkaline phosphatase (calf intestine), pyruvate kinase, phosphoenolpyruvate, adenylate kinase, unlabeled ribo- and deoxyribonucleoside triphosphates, Boehringer Mannheim, Mannheim-Tutting, Germany; Dowex 1-Ci and Stains-all, Serva Feinbiochemica, Heidelberg, Germany; oligo[d(pA)6] and oligo[d(pA)6], P-L Biochemicals Inc., Milwaukee, Wis.; and NCS tissue solubilizer, Amersham/Searle, Arlington Heights, Ill. Herring sperm DNA, isolated according to the method of Zahn et al. (73), was a gift of H. Mack, Illertissen, Germany.

Cell Culture. L5178Y cells were grown in Fisher's medium for leukemic cells (Grand Island Biological Co., Grand Island, N. Y. in suspension culture. Dose-response experiments (inoculation, $5 \times 10^3$ cells/ml) were performed as described previously (38). The cells were incubated at 37° in roller tubes for 72 hr; the controls reached a cell concentration of about $4 \times 10^5$ cells/ml. They needed 120 hr to reach the stationary phase. $ED_50$ was estimated by logit regression. In some experiments, different incubation conditions were used, as described in the text. Cell concentrations and volume distributions were determined with a Model B Coulter counter with a size-distribution plotter (Coulter Electronics, Hialeah, Fla.). In some experiments, the cell proliferation is expressed in doublings (39).

Incorporation Studies. Suspensions (5 ml) of exponentially growing cells at $2 \times 10^4$ cells/ml were supplemented with 3'-dAdo 2 hr prior to the addition of labeled precursor $[\text{H}]d\text{TdThd}$ (25 $\mu$Ci), $[\text{H}]\text{Juridine}$ (25 $\mu$Ci), or $[\text{H}]\text{Lysine}$ (25 $\mu$Ci). The incubations were continued routinely for 30 min. Samples of 5 ml were analyzed for acid-insoluble radioactivity in DNA, RNA, or protein (43). Five hundred-mllm samples, incubated under the same conditions with $[\text{H}]\text{Juridine}$, were used to determine the total uridine pool.

The incorporation studies with $[\text{H}]$3'-dAdo were performed as follows. Exponentially growing cells at $2 \times 10^4$ cells/ml were incubated with 20 $\mu$Ci $[\text{H}$3'-dAdo for 0, 30, 60, or 120 min. Subsequently, the samples were analyzed either for radioactivity in acid-soluble and acid-insoluble (DNA and RNA) fraction (51), for radioactivity in poly(A)-containing and poly(A)-free RNA (68), and for the identification of the labeled compound in the RNA fraction. The sample volumes were 5 ml in the 1st experiment and 100 ml in the latter 2 experiments.

Analysis of RNA. Electrophoresis was carried out on 1-mm-thick 0.7% agarose-3.48% polyacrylamide slab gels. Runs were performed at 24 ma, 100 V, for 135 min at 20° (14). The gels were stained with Stains-all (Serva Feinbiochemica) (15), and the absorbance was monitored continuously at 254 nm. The parallel gels were sliced into 1.4-mm strips, digested in NCS-water-toluene, and counted in aquasol (1).

Isolation of Polyisomes. For the isolation of both membrane-bound and free polyisomes (4) from L5178Y cells on sucrose gradients (0.5 to 1.5 M), procedures previously described (48) were followed with minor modifications (42). One ml of the homogenate contained 0.02 mg DNA, 2.3 mg RNA, and 7.6 mg protein.

Enzyme Preparations. Endoribonuclease IV was isolated and purified nearly to homogeneity from chick oviduct (35); Purification Step 6 with a specific activity of 12,500 units/mg protein was used for the experiments. Phosphotransferase was isolated from wheat seedlings according to the method of Barner and Cohen (3). Exoribonuclease was isolated from mouse liver by the method of Sporn et al. (60); the DEAE fraction with a specific activity of 63 units/mg was used.

DNA-dependent DNA polymerase $\alpha$ was isolated from L5178Y cells according to the method of Rohde et al. (56); the Sephadex-G200 fraction with a specific activity of 65 nmoles of labeled substrate per hr per mg protein was used. DNA-dependent DNA polymerase $\beta$ was isolated from L5178Y cells as described by Chang and Bollum (11); the phosphocellulose fraction with a specific activity of 210 nmoles of nucleotide incorporated per hr per mg protein was taken.

DNA-dependent RNA polymerases I, II, and III were isolated from mouse liver according to the method of Müller et al. (37). The fractions after elution from DEAE-Sephadex were used. The specific activities were: RNA polymerase I, 0.21 nmoles nucleotide incorporated per 15 min per mg protein; enzyme II, 0.36 nmoles per 15 min per mg protein; and enzyme III, 0.09 nmoles per 15 min per mg protein.

Poly(A) polymerase was extracted from quail oviduct according to the method of Müller et al. (41); Fraction I:3 was used. This preparation had a specific activity of 83 nmoles AMP incorporated per 30 min per mg protein.

Terminal riboadenylate transferase was prepared from calf thymus as previously described (67). Fraction V, with a specific activity of 1840 nmoles AMP incorporated per 30 min per mg protein, was used for the studies.

Enzyme Assays. The assay for endoribonuclease IV measured the conversion of high-molecular-weight radioactive poly(A) to low-molecular-weight products. The standard assay mixture (100 $\mu$l) contained 100 mM Tris-HCI, pH 8.7; 0.5 mM MnCl$_2$; 0.2 mM dithiothreitol; bovine serum albumin, 50 $\mu$g/ml; 20 $\mu$l RNA sample, and 0.05 enzyme unit. After incubation at 37° for 0 or 30 min, aliquots of 40 $\mu$l were placed on GF/C filter discs and processed to determine acid-insoluble radioactivity (7).

For the determination of DNA-dependent DNA polymerase activities, 10 $\mu$l enzyme were combined with 50 $\mu$l polymerase $\alpha$ or $\beta$ mixture. The DNA polymerase $\alpha$ mixture consisted of varying amounts of $[\text{H}]d\text{ATP}$ (60 cpm/pmol); 0.1 nmoles of adenosine dCTP, dGTP, and dTTP; 20 mM potassium phosphate buffer (pH 7.2); 1 nmoles mercaptopoethanol; 8 mM MgCl$_2$; and 0.5 A$_{260}$ unit of activated herring sperm DNA. The DNA polymerase $\beta$ mixture was identical with the polymerase $\alpha$ reaction mixture, except that the buffer was 50 mM ammendiol (pH 8.8) rather than potassium phosphate. The reaction was carried out at 37° for 30 min; 50 $\mu$l were placed on GF/C discs and processed as described previously (7).

The standard reaction mixture for RNA polymerases contained the following components in 100 $\mu$l: varying amounts of $[\text{H}]\text{ATP}$ (30 cpm/pmol); 0.1 nmoles each of GTP, CTP, and UTP; 50 mM Tris-HCl, pH 7.8; 3 mM MnCl$_2$; 2 nmoles mercaptopoethanol; 2 nmoles creatine phosphate; creatine phosphokinase, 20 $\mu$g/ml; 5 $\mu$g bovine serum albumin; and 20 $\mu$g native or heat-denatured DNA. The concentrations of (NH$_4$)$_2$SO$_4$ in the incubation mixture for assaying RNA po-

Mode of Action of 3'-d-Ado
lymerase I were adjusted to 50 mm; for Form II and III, 150 mm (NH₄)₂SO₄ was added. Usually, the reaction mixture was incubated at 37° for 15 min after addition of 40 µl of enzyme preparation. After incubation, aliquots of 50 µl were tested on GF/C filters for acid-insoluble radioactivity (7).

The standard assay for poly(A) polymerase (100 µl) contained 100 mm Tris-HCl, pH 8.2; 5 mm MgCl₂; 0.2 mm dithiothreitol; and varying amounts of [³H]ATP (100 cpm/p mole), 15 µg oligo(pA)₁₀, and 20 µl enzyme. After incubation for 30 min at 37°, a 40-µl aliquot was taken on GF/C filters (7) to determine the acid-insoluble radioactivity. The assay to determine terminal riboadenylate transferase contained (36), in a volume of 100 µl, varying amounts of [³H]ATP (30 cpm/p mole); 200 mm Tris-HCl, pH 8.3; 4 mm 2-mercaptoethanol; bovine serum albumin, 20 µg/ml; 0.5 mm MnCl₂; 5 µg oligo(pA)₁₀; and 20 µl enzyme. After incubation, acid-insoluble radioactivity was determined as described above. Some experiments were performed with an oligo(⁶²³°C)pA₁₀ initiator.

Normal and Inhibition Kinetics. For the kinetic experiments to determine the Michaelis constants, concentrations of the labeled ATP in the range between 5 and 35 µM were added to the assays. The inhibitor constants for 3'-dATP were calculated according to the method of Stolzfus et al. (57) with the use of 2 different 3'-dATP concentrations (approximately the Kᵢ value).

Preparation of 3'-dATP. 3'-dAdo was enzymatically phosphorylated to 3'-dAMP by the method of Rottman et al. (57) with the use of the phosphotransferase from wheat sprouts (3). 3'-dAMP was separated from 3'-dAdo and UMP and converted to 3'-dATP by adenylate kinase reaction, as described by Murphy and Lazarrini (44). The [³H]ATP present in the reaction mixture was removed after incubation by periodate oxidation (45); 3'-dATP was subsequently purified by chromatography on Dowex 1-Cl (44). The 3'-dATP preparation was chromatographically pure, as determined by thin-layer chromatography with System 2 (38); no ATP could be detected in the preparation.

Identification of Incorporated 3'-dAdo. Exponentially growing cells (2 × 10⁶ cells/ml) were incubated with 0.53 µM [³H]-3'-dAdo for 120 min. Subsequently, RNA was extracted with chloroform-phenol according to the method of Stoltzfus et al. (61). The RNA fraction obtained was dialyzed for 12 hr at 0° against 10 mm Tris-HCl, pH 7.8. The specific activity was determined at 0.14 × 10⁶ cpm/mg RNA; the RNA concentration was 1.3 mg/ml. Enzymatic digestion of this RNA was performed in a pH-stat (Dosimat; Metrohm, Herisau, Switzerland). First, the RNA was degraded under limiting substrate conditions at 37° and pH 7.8 for 30 min. The reaction mixture contained: RNase A (5 µg), endoribonuclease IV (5 units); exoribonuclease (1 unit); 10 mm Tris-HCl buffer (0.5 mm MnCl₂; 0.2 mm dithiothreitol, pH 7.8); and a 50-µl RNA sample in a volume of 0.1 ml. The produced nucleotides were subsequently dephosphorylated by alkaline phosphatase treatment; 10 µg of this enzyme were added to the reaction mixture, and incubation was continued for 30 min. The nucleosides were analyzed by thin-layer chromatography.

Determination of Uridine Pool. For this analysis we used a modification of the method described by Kraml et al. (31). The sediment, obtained from cells previously incubated with [³H]uridine, was extracted with 5% ice-cold trichloroacetic acid and kept at 0°. After centrifugation, the supernatant was extracted 3 times with 1 volume of diethyl ether and was subsequently brought to pH 7.6 with 5% aqueous ammonia solution. The resulting solution was lyophilized and dissolved in 1 ml of 5% perchloric acid and centrifuged. The supernatant was brought to pH 10.0 by addition of concentrated ammonia solution and was centrifuged again. The new supernatant was lyophilized, and the resulting material was dissolved in isopropyl alcohol-H₂O (10%) and chromatographed on thin-layer chromatography Silica Gel F-254 plates (Merck, Darmstadt, Germany) in the same solution. The urine spot detected by a radiochromatogram scanner was then scraped off. This material was suspended in 2 ml of distilled water, and an aliquot was counted in 10 ml of aquasol, and a 2nd aliquot was used for determination of A₃₂₇₀. The value of the determined radioactivity was a measure for the loss of uridine during the procedures of extraction and purification of this nucleoside; from the absorbance, the concentration of uridine was calculated with the use of the molar extinction coefficient of 10.1 × 10⁴ (59) for uridine.

Miscellaneous Methods. DNA was determined by the method of Kisse and Robins (27), RNA by the orcinol reaction (25), and protein according to the method of Lowry et al. (33). The concentration of poly(A)-containing and that of poly(A)-free RNA were estimated by UV absorption with the use of the extinction coefficient of 25 A₂₆₀/mg RNA (8.05 A₃₂₇₀/µmole of nucleoside monophosphate) (53). Herring sperm DNA was activated by the method of Apohisan and Kornberg (2). Both oligo(pA)₁₀ and oligo(¹⁴C)pA₁₀ were prepared by degradation of poly(A) by endoribonuclease IV (35). The calculation of S values in slab gel was performed according to the method of Cupello and Hydén (14).

The chain length of oligo(A) was determined as previously described (22), except that DE 81 paper was used; 50-µl samples were applied. For spotting the radioactivity, the developed chromatograms were cut into pieces (0.5 × 1.0 cm). Each strip was counted in 5 ml of distilled water. Thin-layer chromatography was performed in an ascending system on thin-layer chromatography Silica Gel F-254 plates (Merck) with a solvent containing water-saturated n-butyl alcohol. The different Rₑ values are: adenosine, 0.29; deoxyadenosine, 0.25; and 3'-dAdo, 0.38. For spotting of the radioactivity, the developed chromatograms were cut into pieces (0.5 × 1.0 cm). Each strip was eluted with 0.3 ml of 0.04 n NaOH under shaking. After centrifugation (12,000 × g for 20 min), 200 µl of the supernatant were used for determination of the radioactivity.

Oligo(¹⁴C)pA₁₀ was separated from 3'-dTTP by Sephadex G-25. A column with a size of 1 x 30 cm was eluted with distilled water, and fractions of 1 ml were collected. The average Rₑ value (17) was 0.06 for the oligo(¹⁴C)pA₁₀ fraction and 0.53 for 3'-dTTP. The oligo(³H)pA fraction was dried under vacuum and dissolved in 50 µl of 0.9% NaCl solution.

RESULTS

The effect of 3'-dAdo was studied both in an intact cell

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Influence of adenosine and deoxyadenosine on cell growth inhibition by 3'-dAdo

Cultures were inoculated with 2 x 10^6 cells/ml and subsequently were incubated in roller tubes for 24 hr at 37°C with 3'-dAdo in the presence or absence of adenosine or deoxyadenosine as indicated. Cell growth is given in doublings. The reduction of cell growth, caused by 3'-dAdo together with the different compounds, was evaluated by subtracting the number of doublings with 3'-dAdo alone from the number of doublings with 3'-dAdo plus adenosine or deoxyadenosine. Each value represents the mean ± S.D. of 10 parallel assays.

<table>
<thead>
<tr>
<th>3'-dAdo (μM)</th>
<th>Additional compound (μM)</th>
<th>Cell concentration after incubation (cells x 10^6/ml)</th>
<th>Cell doublings</th>
<th>Alteration of 3'-dAdo effect by additional compound [increase (+)/decrease (–) in doubling steps]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td></td>
<td>475 ± 42</td>
<td>1.25</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>310 ± 26</td>
<td>0.64</td>
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<tr>
<td>5</td>
<td></td>
<td>230 ± 18</td>
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<td>0</td>
<td>Deoxyadenosine (30)</td>
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<td>1</td>
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<td>470 ± 42</td>
<td>1.23</td>
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<td>2</td>
<td></td>
<td>330 ± 28</td>
<td>0.72</td>
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</tr>
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<td>5</td>
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<td>245 ± 23</td>
<td>0.29</td>
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<tr>
<td>0</td>
<td>Adenosine (10)</td>
<td>515 ± 48</td>
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<tr>
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<tr>
<td>2</td>
<td></td>
<td>410 ± 39</td>
<td>1.04</td>
<td>+0.40</td>
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<tr>
<td>5</td>
<td></td>
<td>320 ± 27</td>
<td>0.68</td>
<td>+0.48</td>
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</table>
Influence of 3'-dAdo on the synthesis of DNA, RNA, and protein in L5178Y cells

The incorporation studies were performed as described under ‘Materials and Methods.’ Exposure time of the precursors was 30 min. Values represent means of 4 parallel experiments. The S.D. does not exceed 10%. The influence of 3'-dAdo on cell proliferation is expressed in doublings.

Table 2

<table>
<thead>
<tr>
<th>3'-dAdo concentration (µM)</th>
<th>Influence on cell doublings</th>
<th>Incorporation into macromolecules/100,000 cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>[3H]dThd</td>
<td>[3H]Urd</td>
</tr>
<tr>
<td>0.00</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>0.03</td>
<td>100</td>
<td>98</td>
</tr>
<tr>
<td>0.06</td>
<td>100</td>
<td>90</td>
</tr>
</tbody>
</table>

Table 3

Influence of 3'-dAdo on the uptake of [3H]Uridine and on total uridine pool in L5178Y cells

Exponentially growing cells were incubated for 120 min with 0 or 5 µM 3'-dAdo. Subsequently, [3H]Uridine, 5 µCi/ml, was added to the medium, and incubation was continued for 30 min. Then 1 aliquot was taken to determine the amount of radioactivity in the acid-soluble fraction; in a 2nd aliquot, the total uridine pool was determined. Values represent means of 4 parallel experiments. The S.D. does not exceed 8%. Further details are given under ‘Materials and Methods.’

Table 4

Distribution of radioactivity in L5178Y cells after incubation with [3H]-3'-dAdo

Exponentially growing cells were incubated with 0.53 µM [3H]-3'-dAdo as described under ‘Materials and Methods.’ Radioactivity in acid-soluble and acid-insoluble fraction (RNA was separated from DNA by alkaline digestion and precipitation with acid) was determined (43).

Table 5

Distribution of radioactivity in L5178Y cells after incubation with [3H]-3'-dAdo

<table>
<thead>
<tr>
<th>Fraction</th>
<th>0 min</th>
<th>30 min</th>
<th>60 min</th>
<th>120 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acid-soluble</td>
<td>70</td>
<td>3940</td>
<td>4100</td>
<td>5600</td>
</tr>
<tr>
<td>DNA</td>
<td>30</td>
<td>10</td>
<td>50</td>
<td>70</td>
</tr>
<tr>
<td>RNA</td>
<td>10</td>
<td>950</td>
<td>2520</td>
<td>4750</td>
</tr>
</tbody>
</table>

from 2 × 10^7 cells; after incubation with 2 or 5 µM 3'-dAdo, the quantity of polysomes amounted to 0.24 A_260 unit (75%) and 0.15 A_260 unit (47%), respectively. From these data we conclude that the relative rate of mRNA synthesis was reduced by 3'-dAdo after the short incubation period of 2 hr.

Incorporation into Nucleic Acids. [3H]-3'-dAdo was incorporated into nucleic acid (Table 4), particularly in the RNA fraction. Under a compound concentration of 0.53 µM, which had no influence on cell proliferation, 4730 cpm (161 pg/10^6 cells) 3'-dAdo were incorporated into the RNA fraction during a 2-hr period; only a minute amount of radioactivity (70 cpm) was found in the DNA fraction. The total RNA content in 10^6 cells was analytically determined (43).

Incorporation studies were performed as described under ‘Materials and Methods.’ Radioactivity in acid-soluble and acid-insoluble fraction (RNA was separated from DNA by alkaline digestion and precipitation with acid) was determined (43).

The specific incorporation rate of [3H]-3'-dAdo was 3.3-fold higher into poly(A)-containing RNA than into poly(A)-free RNA. From this we conclude that after short-term incubation 3'-dAdo is incorporated predominantly into poly(A) sequences. To verify this, the 2 RNA fractions were treated with endonuclease IV. This enzyme has recently been isolated and purified from chick oviduct and had been shown to hydrolyze specifically poly(A) sequences on mRNA (35, 40). As demonstrated in Table 6, this enzyme reduces the acid-insoluble radioactivity of poly(A)-free RNA by only 6%, while it reduces the acid-insoluble radioactivity of poly(A)-containing RNA by 97.4%.

The poly(A)-free from 3'-dAdo-treated and untreated cells was analyzed by slab gel electrophoresis (Chart 1). Under nontoxic conditions (incubation of the cells with 0.53 µM 3'-dAdo for 2 hr), no significant change in the amount of the different RNA species in the controls (Chart 1, top) and in 3'-dAdo-treated cells (middle) could be observed. Four pronounced RNA peaks were found: 28 S (rRNA), 18
Incorporation of [3H]-3'-dAdo into poly(A)-containing RNA and poly(A)-free RNA

Cultures of 100 ml at a cell density of 2 x 10^5/ml were incubated for 2 hr with 0.53 μM [3H]-3'-dAdo (3'-dAdo-treated cultures) or without the compound (controls). Subsequently, the cells were harvested; after extraction of RNA, the poly(A)-containing RNA fraction was isolated by affinity chromatography on poly(U)-Sephrose (column, 0.5 x 4 cm). Both the yield of extraction of the 2 RNA fractions [poly(A) containing and poly(A) free] and their specific radioactivity were determined. The radioactivity of the RNA was counted in 10 ml aquasol in a scintillation counter.

<table>
<thead>
<tr>
<th>Incorporation of [3H]-3'-dAdo into poly(A)-containing RNA and poly(A)-free RNA</th>
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</thead>
<tbody>
<tr>
<td>Fraction</td>
</tr>
<tr>
<td>-----------------------------</td>
</tr>
<tr>
<td>Poly(A)-containing RNA</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Poly(A)-free RNA</td>
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<td></td>
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</table>

Accessibility of [3H]-3'-dAdo-labeled poly(A)-containing and poly(A)-free RNA toward endoribonuclease IV

The 2 RNA fractions were obtained as described in the legend to Table 5 and "Materials and Methods." The concentration of radioactivity was 850,000 cpm/ml in poly(A)-containing RNA and 630,000 cpm/ml in poly(A)-free RNA. The 2 RNA fractions were incubated in the presence or absence of endoribonuclease IV, as described in "Materials and Methods;" acid-insoluble radioactivity was determined.

<table>
<thead>
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<th>Acid-insoluble radioactivity (cpm/assay)</th>
</tr>
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<tbody>
<tr>
<td>Fraction</td>
</tr>
<tr>
<td>Nuclease omitted</td>
</tr>
<tr>
<td>Nuclease added</td>
</tr>
<tr>
<td>Poly(A)-containing RNA</td>
</tr>
<tr>
<td>Poly(A)-free RNA</td>
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</table>

Influence of 3'-dATP on DNA and RNA Polymerases.

The 2 major eukaryotic DNA polymerases (α and β) were tested for their sensitivity 3'-dATP (Table 7). At a substrate (dATP) concentration of 0.06 mM and a 3'-dATP concentration of 0.6 mM, no inhibition of the incorporation rate was observed. The 3 different RNA polymerases (I, II, and III) present in nuclei of eukaryotes were moderately inhibited by 3'-dATP (Table 7). The inhibition was of the competitive type (32) with respect to ATP. As a measure of the relative affinity of the enzyme for 3'-dATP and ATP in competitive inhibition, the ratio K_i/K_m can be adopted (69); the lower the value for K_i/K_m, the stronger is the inhibitory potency of a substance. The highest affinity of 3'-dATP was observed toward DNA-dependent RNA polymerase II (ratio, 1.67); lower ratios were observed in the experiments with RNA polymerase I and III.

The highest inhibitory potency of 3'-dATP was observed in the assays containing ATP:polynucleotidyribonucleoside. According to Tsiapalis et al. (67), this class of enzymes should be divided into nuclear poly(A) polymerase (Mg^2+ dependent) and cytoplasmic terminal riboadenylate transferase (Mn^2+ dependent). The studies to determine the inhibitory potency of 3'-dATP in these enzyme systems revealed (Table 7) that both enzymes were inhibited purely...
Incorporation of \[3H\]dATP and \[3H\]ATP into DNA or RNA was determined, and kinetic constants were evaluated as described in "Materials and Methods." Form of the enzyme

<table>
<thead>
<tr>
<th>Enzyme Source</th>
<th>Template</th>
<th>(K_m) ((\mu)M)</th>
<th>(K_i) ((\mu)M)</th>
<th>(K_i/K_m)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA-dependent DNA polymerase DNA</td>
<td>L5178Y Activated DNA</td>
<td>12.4 ± 2.0</td>
<td>6.3 ± 1.7</td>
<td>No inhibition</td>
</tr>
<tr>
<td>DNA-dependent RNA polymerase Mouse liver Native DNA</td>
<td>16.8 ± 2.9</td>
<td>42.3 ± 4.3</td>
<td>2.52</td>
<td></td>
</tr>
<tr>
<td>DNA-dependent RNA polymerase Mouse liver Denatured DNA</td>
<td>23.9 ± 3.4</td>
<td>39.9 ± 4.1</td>
<td>1.67</td>
<td></td>
</tr>
<tr>
<td>DNA-dependent RNA polymerase Mouse liver Native DNA</td>
<td>27.1 ± 3.7</td>
<td>73.4 ± 6.2</td>
<td>2.71</td>
<td></td>
</tr>
<tr>
<td>Poly(A) polymerase Oviduct Oligo(pA)&lt;sub&gt;10&lt;/sub&gt;</td>
<td>34.7 ± 5.9</td>
<td>7.3 ± 2.3</td>
<td>0.21</td>
<td></td>
</tr>
<tr>
<td>Terminal riboadenylate transferase (Mg&lt;sup&gt;2+&lt;/sup&gt;-dependent) Calf thymus Oligo(pA)&lt;sub&gt;10&lt;/sub&gt;</td>
<td>21.4 ± 3.1</td>
<td>16.0 ± 2.9</td>
<td>0.75</td>
<td></td>
</tr>
</tbody>
</table>

In a series of other experiments the mode of inhibition of poly(A) polymerase by 3'-dATP was studied in more detail. The kinetics in Chart 2 shows that poly(A) polymerase was inhibited by 3'-dATP competitively, not only to the substrate (ATP) but also to the initiator [oligo(pA)<sub>10</sub>]. This result is the 1st indication that 3'-dATP competes with ATP for the active site of the enzyme and then "inactivates" the initiator for the enzyme. The latter function of 3'-dATP to act as chain terminator is demonstrated in the following experiments. After incubation of the oligo([14C]pA)<sub>6</sub> initiator in the presence of poly(A) polymerase and 3'-dATP, the average chain length of the oligomer increased by 1 moiety (Chart 3). This means that poly(A) polymerase did incorporate 1 3'-dAMP moiety on the 3'-hydroxyl terminus of the initiator. The following experiments were intended to clarify whether oligo(pA) with a terminal 3'-dAMP moiety was still an initiator for this enzyme. In Chart 4 it is shown that, after incubation of oligo(pA)<sub>6</sub> with poly(A) polymerase, the average chain length of the oligomer increased considerably; the \(R_b\) value of the initiator oligo(pA)<sub>6</sub> amounted to 0.45 (Chart 3); 84% of the oligomer was characterized after incubation with the enzyme by an average \(R_b\) value of 0.12 (Chart 4), indicating an increase of the chain length of the initiator (22). In contrast to the oligo(pA)<sub>6</sub> initiator, oligo(pA)<sub>6</sub>-3'-AMP was not an initiator for the poly(A) polymerase. After incubation of oligo(pA)<sub>6</sub>-3'-AMP with poly(A) polymerase and ATP, the prevailing amount of radioactivity was found at \(R_b\) 0.4 (Chart 4); this value is identical with that for the unreacted oligo(pA)<sub>6</sub>-3'-AMP (Chart 3). Only 15% of the total radioactivity was found at an \(R_b\) value smaller than 0.2; these oligo(pA) species with a greater chain length than 7 AMP moieties were probably formed by the enzyme from oligo(pA)\(_6\) that had not reacted with 3'-dATP. In conclusion, these data show that 3'-dATP acted as "substrate" for poly(A) polymerase; the resulting product, poly(A)-3'-dAMP, had no more initiator capacity for this enzyme. In other words, incorporated 3'-dAMP acted as chain terminator.

**DISCUSSION**

3'-dAdo is a strong inhibitor of cell proliferation of L5178Y mouse lymphoma cells. The ED<sub>50</sub> of 3'-dAdo has been determined to be 0.27 \(\mu\)M; under comparable culture conditions, the value for the antitumor drug is 0.10 \(\mu\)M for 1-β-D-arabinofuranosylcytosine and 2.9 \(\mu\)M for 9-β-D-arabinofuranosyladenine (38). The inhibitory effect of 3'-dAdo could be abolished by adenosine but not by 2'-deoxyadenosine. This result confirms earlier data (28); in this earlier report, the author suggests that the preventing effect is due to an inhibition of phosphorylation of 3'-dAdo. Our data indicate that 3'-dAdo competes with adenosine for the nucleoside and/or nucleotide phosphorylating enzymes.

In mouse lymphoma cells, 3'-dAdo blocked the overall RNA synthesis selectively without affecting DNA synthesis. This finding is in accordance with a previous report by...
fold higher in poly(A)-containing RNA than in poly(A)-free RNA; we did not determine whether this effect reflects a differential rate of synthesis of the respective RNA species at the same affinity of 3'-dATP to the enzyme systems by which they are synthesized or whether it is due to a selectivity of 3'-dATP toward a certain enzyme system. In previous investigations it was established (for a survey, see Ref. 46) that the incorporated 3'-dAMP moieties in the poly(A)-containing RNA are present in the poly(A) stretch and there, most likely, in terminal positions. Therefore, it can be assumed that incorporated 3'-dAMP moieties in poly(A) segments caused premature termination of the growing poly(A) chain in heterogeneous nuclear RNA and/or mRNA. Concerning poly(A)-free RNA, incorporated 3'-dAMP moieties were present in 28 S RNA, 10 S RNA, 5 S RNA, and 4 S RNA. Only minute amounts of 3'-AMP were found in 18 S RNA. It is well established (71) that 18 S and 28 S RNA in mammals derive from a common 45 S precursor by a series of cleavages. Therefore, we conclude that the 28 S RNA stretch is present in the precursor at the 3'-hydroxyl terminus. In the region of 10 S RNA, a large amount of incorporated 3'-dAMP moieties could be traced. RNA of this size belongs to the class of mRNA. The 3'-dAMP-containing RNA molecules did not bind to poly(U)-Sepharose, indicating a lack of poly(A) sequences.

Two explanations of this finding are possible. Either the mRNA molecules were poly(A) free, like histone RNA (9), or the presence of 3'-dATP caused an inhibition of polyadenylation of poly(A)-mRNA during the initiation phase, as already suggested (8). The observed incorporation of 3'-dAMP into low-molecular-weight RNA (5 S rRNA and 4 S tRNA) was substantial; however, it seems likely that among this low-molecular-weight RNA, not only 5 S rRNA and 4 S tRNA but also premature RNA fragments belonging to higher-molecular-weight RNA classes were present.

In a 3rd approach aimed at elucidating the effect(s) of 3'-dAdo on RNA metabolism, the influence of the drug on the synthesis of poly(A) RNA was studied. Poly(A) RNA was isolated from the 3' distal end of poly(A) RNA molecules by affinity chromatography on DE 81 paper. ---, distribution of the radioactivity of the product after incubation with the oligo[32P]P(A) before and after cleavage with 3'-dAMP-3'-AMP initiator.

Chart 3. Chain length of oligo(pA)6 after incubation with poly(A) polymerase and 3'-dATP. Oligo[32P]P(A)6 in a concentration of 3 µg (15 × 103 cpm) assay (0.1 ml) was incubated in the absence or presence of 0.1 µg 3'-dATP and 40 µl (18 µg) poly(A) polymerase in a 100 µl Tris-HCl buffer (pH 8.2) containing 5 mM MgCl2, 50 µg bovine serum albumin, and 0.2 mM dithiothreitol. After incubation for 60 min at 37°, oligo[32P]P(A)6 was separated from 3'-dATP by gel chromatography, as described in "Materials and Methods." The purified oligo[32P]P(A)6 preparations, 3'-dATP-treated (- - - -) and untreated (-----), were analyzed by paper chromatography, as described in "Materials and Methods." The positions of the authentic compounds.
synthesis of the different RNA species was determined. Frederiksen and Klenow (21) and Truman and Frederiksen (65) have shown that 3′-dAdo does not influence RNA synthesis uniformly. While the 50 to 60 S rRNA was only slightly inhibited, the 28 S rRNA peak was inhibited to 80%, the 18 S peak to 60%, and the 4 to 5 S peak to only 40%. Penman et al. (50) have speculated that the differential response of RNA synthesis to 3′-dAdo might be due to the existence of several distinct polymerases with different sensitivities. In our studies, with the use of low 3′-dAdo concentrations at which the compound was incorporated into RNA without influencing cell proliferation, no significant change in the amount of the different RNA species could be observed. A short application of higher concentrations, however, caused a drastic reduction of the amount of polysomes, indicating a strong influence of 3′-dAdo on the availability of mRNA in the cytoplasm. Further studies are needed to show whether this effect was caused by an inhibition of poly(A) synthesis of nuclear RNA chains or by a suppression of the transcription of mRNA precursor.

In the 4th attempt to clarify the 3′-dAdo effect, the inhibitory potency of 3′-dATP which is synthesized from 3′-dAdo in intact cell systems (29) on a variety of DNA and RNA polymerases responsible for the synthesis of the different nucleic acid species was determined. In earlier reports (5, 24, 34, 63, 66, 70), it was established that 3′-dATP inhibits DNA polymerase I (E. coli), RNA polymerase (Micrococcus lysodeikticus, Ehrlich ascites cells, yeast, E. coli, and maize), poly(A) polymerase (HeLa and maize), and RNA polymerase I and II (Novikoff hepatoma cells) in a competitive way with respect to dATP or ATP. However, kinetic studies to determine the $K_i$ for 3′-dATP and the $K_m$ for ATP in RNA-synthesising systems have been described in only 2 papers (16, 24). The results with the RNA polymerases from mammalian cells revealed (16) that the relative affinity ($K_i/K_m$) of 3′-dATP for polymerase II is 2.3-fold higher than that for polymerase I. In the present study, RNA polymerase II from mouse lymphoma cells was also found to be more sensitively inhibited by 3′-dATP than the Form I and III enzymes. These 3 enzymes have special functions: RNA polymerase I is responsible for the synthesis of ribosomal precursor RNA (52); RNA polymerase II is responsible for the synthesis of precursor mRNA (5); and RNA polymerase III is responsible for 5 S rRNA and tRNA (70). From these findings a preferential inhibition of precursor mRNA synthesis by 3′-dATP could be assumed. However, by far the most sensitively inhibited polymerase systems are the 2 poly(A)-synthesizing enzymes. Comparative studies have shown that the nuclear poly(A) polymerase and the cytoplasmic terminal riboadenylate transferase are 8.0-fold and 2.2-fold, respectively, more sensitively inhibited by 3′-dATP than was the RNA polymerase II. Thus, from the in vitro studies with isolated enzyme systems, poly(A) synthesis is preferentially affected. In contrast to previously published data (63), we found that the DNA polymerases α and β from mouse lymphoma cells were insensitive toward 3′-dATP. In a more recent study (62), some restrictions concerning the observed inhibition of the DNA polymerase I (E. coli) were stated.

Our enzymatic studies were performed with enzymes other than those isolated from L5178Y cells. However, until now there has been no evidence that the different animal DNA-dependent RNA polymerases I to III are also characterized by a different structure, function, or substrate specificity (10). No clear-cut evidence could be presented (18) on the possible presence of multiple animal poly(A) polymerases. Therefore, at this stage of our knowledge, an extrapolation of RNA and poly(A) polymerases from mouse liver, quail oviduct, and calf thymus to those from L5178Y cells seems justified.

From the reported data no final conclusion about the mode of action of 3′-dAdo can be drawn. Nevertheless, the data available suggest that after short incubation of cells with this compound the appearance of mRNA in cytoplasm, most likely via inhibition of poly(A) polymerase(s), is affected. With a longer incubation of 3′-dAdo, the synthesis of other RNA species was reduced. The observed inhibition of DNA and protein synthesis after long-term incubation with 3′-dAdo was most probably a consequence of diminished mRNA synthesis.

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