Structure–Activity Relationship of Some Purine 3′-Deoxyribonucleosides*

Harold T. Shigeura, George E. Boxer, Mary Lou Meloni, and Susan D. Sampson

ABSTRACT: The metabolism of several nucleosides structurally related to 3′-deoxyadenosine by intact Ehrlich ascites cells has been studied. The nucleosides were not cleaved by whole ascites cells. 6-Methylamino-9-(3′-deoxy-β-D-ribofuranosyl)purine was converted to 5′-monophosphate while nucleosides containing *N*-dimethyl and *N*-ethyl groups on C_6 of the purine moiety were not phosphorylated. 3′-Amino-3′-deoxyadenosine was metabolized to 5′-mono-, 5′-di-, and 5′-triphosphate and inhibited the biosynthesis of nucleic acids in whole Ehrlich ascites cells. The 5′-triphosphate derivative of the nucleoside was found to inhibit the activity of deoxyribonucleic acid (DNA) dependent ribonucleic acid (RNA) polymerase obtained from *Micrococcus lysodeikticus*.

6-Dimethyl-3′-amino-3′-deoxyadenosine, a structurally related nucleoside, was not phosphorylated by Ehrlich ascites cells and did not significantly affect nucleic acid synthesis in these cells. A direct correlation between the extent of phosphorylation of the nucleosides and inhibition of ribonucleic acid synthesis has been shown.

3′-Deoxyadenosine, a potent inhibitor of ribonucleic acid (RNA) synthesis in intact Ehrlich ascites cells, was found to be metabolized to the 5′-mono-, 5′-di-, and 5′-triphosphate by these cells (Klenow, 1963a; Shigeura and Gordon, 1965). Furthermore, 3′-deoxyadenosine 5′-triphosphate markedly suppressed the activity of deoxyribonucleic acid (DNA) dependent RNA polymerase obtained from Ehrlich ascites cells (Klenow and Frederickson, 1964) and from *Micrococcus lysodeikticus* (Shigeura and Boxer, 1964; Shigeura and Gordon, 1965). The inhibitory activity of 3′-deoxyadenosine 5′-triphosphate thus appeared to account for part of the inhibition by 3′-deoxyadenosine of RNA synthesis in whole ascites cells.

Although 3′-deoxyadenosine inhibited nucleic acid synthesis in Ehrlich ascites cells and also in *Bacillus subtilis* (Rottman and Guarino, 1964), it has been found to be inactive against a number of other bacterial and mammalian cells presumably due to the action of adenosine deaminase. 3′-Deoxyinosine, a product of this reaction, has been shown to be inactive in Ehrlich ascites cells (Klenow, 1963b). These observations suggested that structural modifications of 3′-deoxyadenosine at the 6-amino position may provide more stable and consequently more active inhibitors than the parent compound. With this in mind, several compounds structurally related to 3′-deoxyadenosine have now been synthesized in these laboratories (Walton *et al.*, 1965). The effects of these new nucleosides on RNA synthesis in whole Ehrlich ascites cells and possible correlation between structure and activity of these compounds are discussed in this report.

The metabolism of 3′-amino-3′-deoxyadenosine, originally synthesized by Baker *et al.* (1955) and subsequently isolated from *Helminthosporium* sp. no. 215 by Pugh and Gerber (1963), was also investigated to determine the effect of structural alteration of the deoxyribose moiety on phosphorylation. Concurrent experiments were done with a structurally related compound, puromycin aminonucleoside, in which the 6-amino group is replaced by the 6-dimethylamino moiety.

Experimental Procedure

Materials. Tritiated 6-methylamino-9-(3′-deoxy-β-D-ribofuranosyl)purine and all unlabeled nucleosides structurally related to 3′-deoxyadenosine were synthesized in these laboratories by Walton *et al.* (1965). 6-Methylaminopurine and 6-methylamino-9-(β-D-ribofuranosyl)purine were purchased from Cyclo Chemical Corp. ^{14}C-Labeled chemicals were purchased from Nuclear-Chicago Corp. and Schwarz BioResearch, Inc. Unlabeled hypoxanthine, adenine, adenosine, inosine, uridine, and xanthosine were obtained from Calbiochem. Spermidine phosphate, calf-thymus DNA, puromycin aminonucleoside, and spray-dried cells of *M. lysodeikticus* were purchased from Mann Research Laboratories, Sigma Chemical Co., Nutritional Biochemicals Corp., and Miles Chemical Co., respectively. 3′-Amino-3′-deoxyadenosine was generously supplied by Dr. Nancy N. Gerber, Institute of Microbiology, Rutgers University, New Brunswick, N. J.

Experiments with Whole Ehrlich Ascites Cells. A. The procedure used to examine the effects of various compounds on nucleic acid synthesis in intact ascites cells

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has been described in detail in previous reports (Shigeura and Gordon, 1962, 1965). A brief description of the method is given in the legend to Table I.

B. To test the effects of nucleosides on the synthesis of 5-phosphoribosylamine in whole cells, the method described by Henderson (1962) was used except for the substitution of Robinson's medium (1949) for Krebs–Ringer's medium. The reaction mixture in a final volume of 2.1 ml contained 5% ascites cells, 2.5 mM uniformly labeled glycine-^{14}C (67 μc/μmole), 1.8 mM glutamine, 10 μg of azaserine, and 1.0 mM nucleoside. After shaking for 1 hr at 37°, 0.25 ml of cold 4 M $HClO_4$ was added and the mixture was centrifuged. The supernatant fluid after neutralization with KOH was applied on a 0.6 × 18 cm Dowex-1 formate column and eluted with 50 ml each of 0.5 and 4.0 M formic acid. The 4.0 M formic acid eluate containing α-*N*-formylglycinamide-ribonucleotide was concentrated *in vacuo* to 3 ml and total radioactivity was measured. The percentage of total activity obtained as compared to that in the control experiment was taken as the measure of feedback inhibition of purine biosynthesis by the test nucleoside.

TABLE I: Effects of Purine 3′-Deoxyribonucleosides on the Incorporation of Hypoxanthine-8-^{14}C into Nucleic Acids in Whole Ehrlich Ascites Cells.[a]

Expt No.	Compd	mM	% Inhib
1	6-Methylamino-9-(3′-deoxy-β-D-ribofuranosyl)purine	2.0	30
2	6-Dimethylamino-9-(3′-deoxy-β-D-ribofuranosyl)purine	2.0	25
3	6-Ethylamino-9-(3′-deoxy-β-D-ribofuranosyl)purine	2.0	15
4	Purine 3′-deoxyribonucleoside	2.0	20
5	2,6-Diamino-9-(3′-deoxy-β-D-ribofuranosyl)purine	2.0	8
6	3′-Deoxyadenosine	0.5	95
7	3′-Amino-3′-deoxyadenosine	0.5	57
8	Puromycin aminonucleoside	1.5	18

[a] Ehrlich ascites cell suspensions (3.8 ml; 6–9 × 10^7 cells/ml) in Robinson's medium (Robinson, 1949) contained 0.02 M $KHCO_3$, 0.1% glucose, hypoxanthine-8-^{14}C (0.5 μc/0.084 μmole), and indicated amounts of test compound. The mixtures were shaken at 37°. After 30 min, 5 ml of cold 5% TCA was added. The acid-insoluble precipitate was washed with cold 5% TCA, absolute alcohol, and alcohol–ether, and dried. Nucleic acid was isolated by extraction in hot 10% NaCl and measured for radioactivity. The percentage inhibition as compared to control experiment was calculated.

C. LePage and Junga (1965) have shown that 3′-deoxyadenosine (cordycepin) was not cleaved by either hydrolysis or phosphorolysis into adenine and 3′-deoxyribose by ascites tumor cells. The possible cleavage of 3′-deoxyadenosine congeners by Ehrlich ascites cells was examined as follows: The reaction mixture in a total volume of 2.0 ml contained 5% ascites cells in Robinson's medium (without glucose), 0.02 M $KHCO_3$, and 0.002 M nucleoside. After shaking for 1 hr at 37°, 0.22 ml of ice-cold 4 M $HClO_4$ was added and the mixture was chilled. After centrifugation, the clear supernatant fluid was neutralized. Appropriate aliquots of the supernatant fluid were applied on Whatman No. 3MM papers and developed with a two-phase solvent system containing equal parts by volume of isoamyl alcohol and 5% sodium phosphate (pH 7.0) for 8–8.5 hr (Carter, 1950). The ultraviolet-absorbing zones were located, cut into 0.5-in. strips, eluted with water, and measured for radioactivity in a liquid scintillation counter or for ultraviolet absorption with a Cary recording spectrophotometer.

D. To examine the possible phosphorylation of nucleosides by intact ascites cells, the method used by Klenow (1963a) was slightly modified as follows: The reaction mixture in a volume of 20 ml of Robinson's medium contained 3.2 ml of packed ascites cells, 0.6% glucose, 119 mg of sodium succinate·$2H_2O$, and 10 mg of test nucleoside. In some experiments, hypoxanthine-8-^{14}C (5 × 10^5 cpm, 0.25 μmole) was added. After shaking for 3 hr at 37°, the reaction mixture was chilled and centrifuged. The perchloric acid soluble fraction of the cells was neutralized with dilute KOH and applied on a column (0.6 × 18 cm) of Dowex-1 formate. H_2O, 1.1 M, and 2.2 M ammonium formate (300 ml each) were placed in successive chambers of a gradient mixer and 5-ml eluates were collected at a rate of about 0.3 ml/min. Approximately 100 fractions were collected for each experiment. In experiments where hypoxanthine-8-^{14}C was used, 0.2-ml aliquots of the eluates were removed and radioactivity was determined in a scintillation counter. The positions of the fractions designated by letters A–G (Figure 2) varied slightly from experiment to experiment. The major ultraviolet-absorbing zones were pooled, adsorbed on charcoal, eluted with 50% ethanol containing 0.1% NH_4OH, and concentrated *in vacuo* to dryness. The residue was dissolved in 3 ml of H_2O and stored at −18° until used for characterization.

RNA Polymerase. The preparation of RNA polymerase from *M. lysodeikticus* and the methods used for enzyme assay were the same as those reported by Nakamoto *et al.* (1964) and Fox and Weiss (1964).

Analytical Methods. Phosphorus was determined by the method of Fiske and Subbarow (1925), periodate oxidation by a spectrophotometric method (Dixon and Lipkin, 1954), ribose by the orcinol method, and anthrone reaction according to the method of Kredich and Guarino (1960). Paper electrophoresis was done in 0.05 M citrate buffer (pH 3.5) at 20 v/cm and the ultraviolet light absorbing substances were extracted with H_2O and characterized.

TABLE II: The Effects of 3′-Amino-3′-deoxyadenosine on the Incorporation of Hypoxanthine-8-^{14}C or Orotic Acid-6-^{14}C into RNA in Whole Ascites Cells.[a]

Labeled Compd	3′-Amino-3′-deoxyadenosine (mM)	AMP-2′,3′-PO$_4$ Control	Test	% Inhib	GMP-2′,3′-PO$_4$ Control	Test	% Inhib
Hypoxanthine, 0.5 μc/0.084 μmole	0.9	1850[b]	317	83	246	104	58
		UMP-2′,3′-PO$_4$					
		Control	Test	% Inhib			
Orotic acid, 2.25 μc/0.75 μmole	0.9	5820	2690	54			

[a] Ascites cell suspensions (4.2 ml; 6–9 × 10^7 cells/ml) in Robinson's medium (1949) containing 0.02 M $KHCO_3$, 0.1% glucose, and indicated amounts of radioactive compounds were incubated with or without 3′-amino-3′-deoxyadenosine. The mixtures were shaken for 30 min at 37°. After precipitation with cold 0.4 M $HClO_4$, the nucleotides obtained from the acid-insoluble fractions were separated by Dowex-1 formate chromatography and measured for radioactivity. [b] Cpm/μmole of nucleotide.

Results

Whole Ehrlich Ascites Cells. The effects of several purine 3′-deoxyribonucleosides on the incorporation of hypoxanthine-8-^{14}C into total nucleic acids in whole ascites cells are shown in Table I. 3′-Deoxyadenosine, previously shown to be a potent inhibitor in this system (Shigeura and Gordon, 1965), was included for comparison (expt 6). It can be seen that structural alteration of 3′-deoxyadenosine by either complete removal of the 6-amino group (4) or substitution of the hydrogen atom by alkyl groups (1–3) resulted in drastically reduced activity. Similarly, no significant inhibition was observed when 2,6-diaminopurine was substituted for adenine in the 3′-deoxyribonucleoside (5).

3′-Amino-3′-deoxyadenosine, although slightly less active than 3′-deoxyadenosine, was considerably more active than puromycin aminonucleoside. It was also found that the incorporation of hypoxanthine-8-^{14}C into adenylic and guanylic acids and that of orotic acid-6-^{14}C into uridylic acid from RNA were markedly suppressed by 3′-amino-3′-deoxyadenosine (Table II).

The effects of various nucleosides on the synthesis of 5-phosphoribosylamine in intact ascites cells are shown in Table III. 3′-Deoxyadenosine and 2-fluoroadenosine, nucleosides previously shown to be phosphorylated to the 5′-triphosphate level (Shigeura and Gordon, 1965; Shigeura *et al.*, 1965), inhibited the reaction by 92 and 94%, respectively. Substitution of a methyl group at the 6-amino position resulted in lower activity [52% as compared to 92% for 3′-deoxyadenosine (5 *vs.* 2)]. 6-Methylamino-9-(β-D-ribofuranosyl)purine (4) was about as active as 6-methylamino-9-(3′-deoxy-β-D-ribofuranosyl)purine. 3′-Amino-3′-deoxyadenosine and 6-dimethyl-3′-amino-3′-deoxyadenosine inhibited the reaction by 74 and 24%, respectively. The remaining congeners of 3′-deoxyadenosine (6–8) were essentially inactive. The effects of adenosine, uridine, and xanthosine were included for comparative purposes.

TABLE III: Effects of Nucleosides on the Synthesis of 5-Phosphoribosylamine in Whole Ascites Cells.[a]

Expt No.	Compd	% Inhib
1	Adenosine	70
2	3′-Deoxyadenosine	92
3	2-Fluoroadenosine	94
4	6-Methylamino-9-(β-D-ribofuranosyl)purine	62
5	6-Methylamino-9-(3′-deoxy-β-D-ribofuranosyl)purine	52
6	6-Dimethylamino-9-(3′-deoxy-β-D-ribofuranosyl)purine	9
7	6-Ethylamino-9-(3′-deoxy-β-D-ribofuranosyl)purine	7
8	2,6-Diamino-9-(3′-deoxy-β-D-ribofuranosyl)purine	14
9	3′-Amino-3′-deoxyadenosine	74
10	6-Dimethyl-3′-amino-3′-deoxyadenosine	24
11	Uridine	14
12	Xanthosine	8

[a] The concentration of nucleosides was 1.0 mM.

The result of an experiment demonstrating that tritiated 6-methylamino-9-(3′-deoxy-β-D-ribofuranosyl)-purine was not cleaved during incubation with whole Ehrlich ascites cells is shown in Figure 1 (lane 2). The predominant ultraviolet-absorbing zones on the paper strips are enclosed with heavy lines and weakly absorbing zones by dotted lines. The major ultraviolet-absorbing zone in lane 2 corresponded to authentic 6-methylamino-9-(3′-deoxy-β-D-ribofuranosyl)purine

with R_F value of 0.64 (Table IV) and essentially all of the radioactivity was associated with the nucleoside. No ultraviolet-absorbing material or radioactivity was found in the area corresponding to 6-methylamino-purine (R_F 0.48). For comparative purposes, inosine-8-^{14}C was treated in the same manner. In agreement with the work of Gotto *et al.* (1964), this nucleoside was partially cleaved (lane 3). The major ultraviolet-absorbing zones corresponded to those of authentic hypoxanthine and inosine (Table IV). The faintly ultraviolet-absorbing materials (presumably containing nucleotides) in the control (lane 1) and those in the experimental strips were not characterized.

TABLE IV: R_F Values of Compounds Obtained from Paper Chromatograms.

Expt No.	Compd	Obsd R_F[a]	R_F of Authentic Compd
1	Hypoxanthine		0.62
2	Adenine		0.40
3	6-Methylaminopurine		0.48
4	6-Methylamino-9-(β-D-ribofuranosyl)purine	0.65	0.65
5	6-Methylamino-9-(3′-deoxy-β-D-ribofuranosyl)purine	0.64	0.65
6	6-Ethylamino-9-(3′-deoxy-β-D-ribofuranosyl)purine	0.68	0.69
7	3′-Deoxyadenosine	0.53	0.52
8	3′-Amino-3′-deoxyadenosine	0.58	0.58
9	Inosine	0.75, 0.61[b]	0.75
10	Adenosine	0.55	0.56

[a] Nucleosides incubated with intact Ehrlich ascites cells are listed on left (no. 4–9). The R_F values of ultraviolet-absorbing zones on paper chromatograms are listed under Obsd R_F. [b] Spot corresponding to hypoxanthine.

As in the case of 6-methylamino-9-(3′-deoxy-β-D-ribofuranosyl)purine, 6-ethylamino-9-(3′-deoxy-β-D-ribofuranosyl)purine, and 6-methylamino-9-(β-D-ribofuranosyl)purine were also not cleaved (Table IV). No ultraviolet-absorbing materials were found in the areas corresponding to the free bases. The R_F values of the predominant ultraviolet-absorbing zones obtained experimentally with various nucleosides are indicated under Obsd R_F. Except in the case of inosine, only one ultraviolet-absorbing zone was seen in each experiment and these were found to correspond to the added nucleoside by R_F values and absorption spectra.

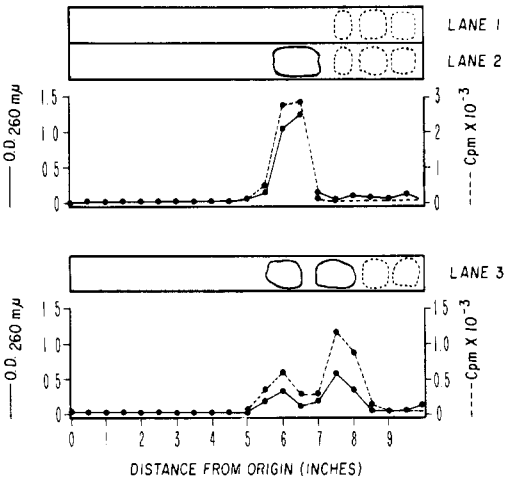

FIGURE 1: Distribution of radioactive and ultraviolet-absorbing substances on paper chromatograms. Lane 1, control. Lanes 2 and 3, perchloric acid soluble materials after incubation with tritiated N^6-methylamino-9-(3′-deoxy-β-D-ribofuranosyl)purine and inosine-8-^{14}C, respectively. The graphs below lanes 2 and 3 show the relative amounts of radioactive and ultraviolet-absorbing substances eluted from the corresponding paper chromatograms.

Since the inhibitory activity of 3′-deoxyadenosine in Ehrlich ascites cells has been shown to be related to the formation of phosphorylated derivatives of the nucleoside (Klenow, 1963a; Shigeura and Gordon, 1965), the possibility that the low level of activity of the structural analogs shown in Tables I–III was due mainly to the absence of phosphorylation by ascites cells was next studied.

As reported previously (Klenow, 1963a; Shigeura and Gordon, 1965), when whole Ehrlich ascites cells were incubated with 3′-deoxyadenosine for 3 hr at 37°, 3′-deoxyadenosine 5′-mono-, 5′-di-, and 5′-triphosphate were formed and could be isolated from the perchloric acid soluble fraction of the cells. For the purpose of comparison with other nucleosides, the experiment with 3′-deoxyadenosine was repeated and the elution pattern of the acid-soluble fraction is shown in Figure 2 (expt 1). Fractions C, D, and F were characterized as the 5′-mono-, 5′-di-, and 5′-triphosphate of 3′-deoxyadenosine (Table V; also see Shigeura and Gordon, 1965). The relative proportion of phosphorylated derivatives varied from experiment to experiment. In other experiments done under identical conditions, approximately equal quantities of the three phosphorylated compounds were obtained. The ultraviolet-absorbing substances in zones A, B, and E were not characterized. It should be noted that no labeled adenosine nucleotides were formed from hypoxanthine-8-^{14}C, an observation consistent with the fact that 3′-deoxyadenosine 5′-triphosphate inhibited the synthesis of 5-phosphoribosyl pyrophosphate in Ehrlich ascites cells (Overgaard-Hansen, 1964).

TABLE V: Properties of Nucleosides and Fractions Obtained from Dowex-1 Formate Chromatography.

Expt No.[a]	Nucleoside	Chromatography Fraction	Spectra at pH 6.7						P/base	Periodate[b] Consumed	Yield (μmoles)
			λ_{min} (mμ)	λ_{max} (mμ)	ϵ_{max}	250/260	280/260	290/260			
1	3′-Deoxyadenosine		228	259	15,400	0.77	0.15	0.01			
		C	228	260		0.76	0.17	0.05	1.29	0.04	
		D	228	260		0.76	0.14	0.01	2.04	0.04	
		F	227	259		0.83	0.15	0.02	2.90	0.10	7.84
2	Adenosine		227	258	15,400	0.78	0.14	0.00			
		D	230	259		0.76	0.17	0.02	2.19		
		F	229	259		0.80	0.17	0.01	3.12		
3	Control	D	226	260		0.76	0.16	0.02	2.10		0.24
		F	230	258		0.80	0.19	0.03	3.02	1.02	1.92
4	6-Methylamino-9-(3′-deoxy-β-D-ribofuranosyl)-purine		232	266	15,900	0.57	0.68	0.22			
		C	232	265		0.59	0.64	0.20	1.15		1.07
		F	230	258		0.80	0.19	0.03	2.75	1.20	1.35
5	Tritiated 6-methylamino-9-(3′-deoxy-β-D-ribofuranosyl)-purine		232	266	15,900	0.57	0.68	0.22			
		C	232	265		0.58	0.67	0.20	1.31		
		F	230	259		0.79	0.20	0.02	3.33		
6	6-Dimethylamino-9-(3′-deoxy-β-D-ribofuranosyl)purine		235	276	18,300	0.46	1.55	0.96			
		F	230	259		0.80	0.22	0.05	2.93	1.06	1.16
7	6-Ethylamino-9-(3′-deoxy-β-D-ribofuranosyl)purine		230	267	16,800	0.53	0.74	0.24			
		F	229	260		0.80	0.20	0.03	2.95	1.00	2.27
8	6-Methylamino-9-(β-D-ribofuranosyl)-purine		232	266	16,900	0.56	0.67	0.24			
		C	232	266		0.59	0.66	0.23	1.07		
		F	228	259		0.80	0.19	0.04	3.14		
9	2,6-Diamino-9-(3′-deoxy-β-D-ribofuranosyl)purine		236, 365	250, 280	9,300	1.02	1.19	0.82			
		F	227	259		0.80	0.17	0.02	3.10		2.82
		G	230	249		1.25	0.68	0.31	2.77		0.54

[a] Nucleoside (10 mg) was used in each experiment. [b] Indicates micromoles of periodate consumed per micromole of nucleotide.

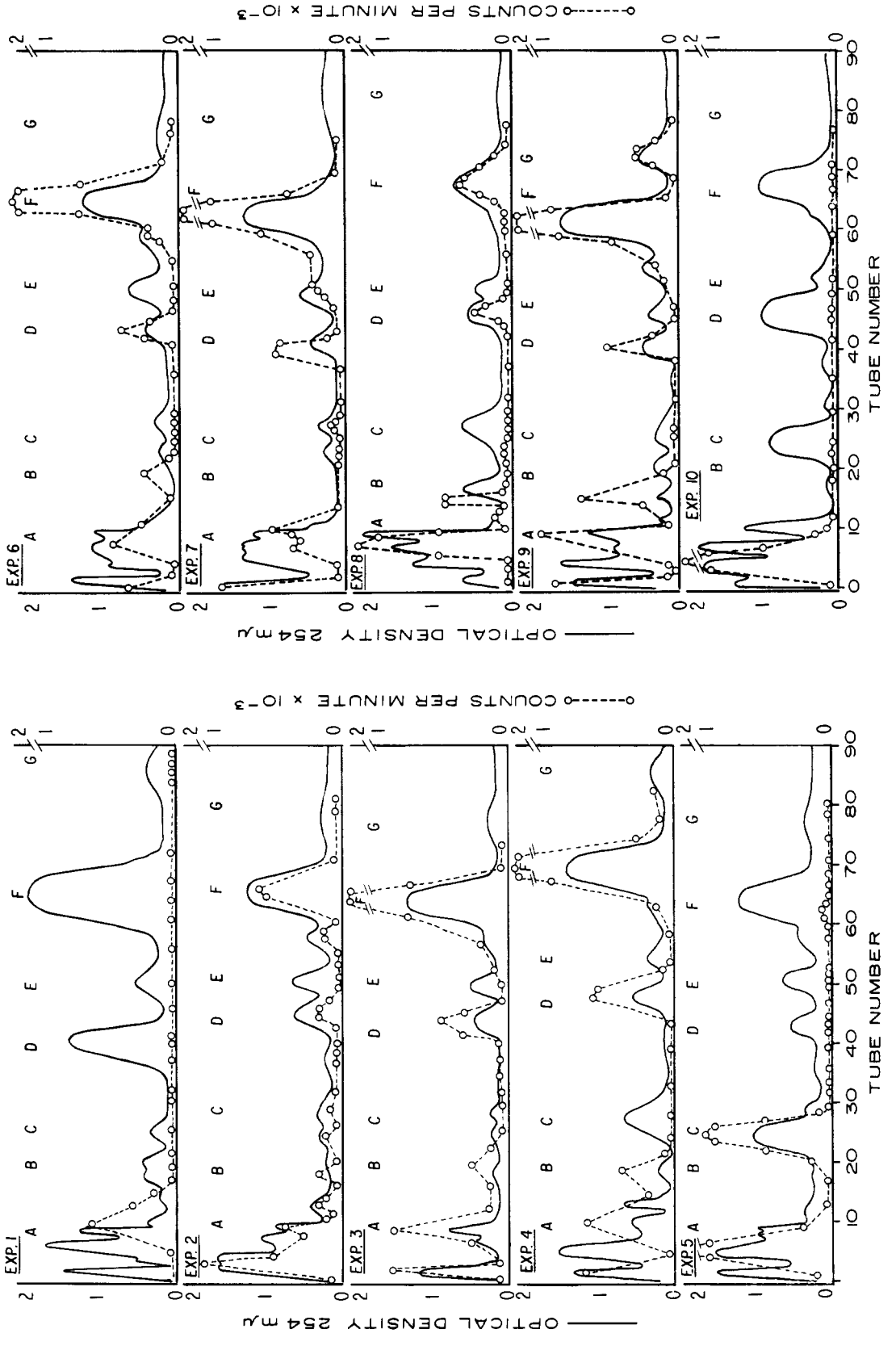

FIGURE 2: Chromatographic elution patterns of perchloric acid solution fractions of ascites cells incubated with indicated nucleosides. See text for details.

The result of an experiment in which adenosine-8-^{14}C was incubated with ascites cells is shown in expt 2. Adenosine was metabolized to the 5′-triphosphate (F). Under these conditions, only a trace of adenosine monophosphate (AMP)[1] appeared to be present (tubes 20–25). Fraction D appeared to be adenosine diphosphate (ADP) by its absorption spectrum and phosphorus analysis. When subjected to paper electrophoresis (pH 3.5), fraction D displayed the same electrophoretic mobility as authentic ADP. The radioactive materials in fractions A and B were not characterized.

In preliminary incubation experiments with 6-*N*-alkylated congeners of 3′-deoxyribonucleosides, it was observed that a large amount of ultraviolet light absorbing material was eluted between tubes no. 60 and 70. This material was unexpectedly found to be adenosine triphosphate (ATP). No 5′-triphosphates of the 6-*N*-alkylated nucleosides were detected in these experiments. Subsequently, in order to aid in locating ATP and other adenosine derivatives, trace amounts (1.7 μg/ml) of hypoxanthine-8-^{14}C were included in most of the experiments. A control experiment in which ascites cells were incubated in a complete medium without nucleosides is shown in expt 3. Radioactive fraction F was characterized as ATP (Table V) by absorption spectroscopy, phosphorus analysis, periodate oxidation, and paper electrophoresis (pH 3.5) with authentic ATP used as marker. Fraction D, upon purification with charcoal, resembled ADP by ultraviolet absorption and phosphorus determination. The material, upon subjection to paper electrophoresis, showed the same mobility as authentic ADP. In all subsequent studies, except expt 10, fractions D and F were characterized as ADP and ATP, respectively (Table V). Radioactive fractions A and B were not purified but may be nicotinamide–adenine dinucleotide (NAD), nicotinamide–adenine phosphate (NADP), and AMP, respectively. The unlabeled fractions (C and E) were assumed to be pyrimidine nucleotides. In some of the experiments, fraction G was characterized as guanosine triphosphate (GTP). In a parallel experiment (not shown in figure) in which hypoxanthine-8-^{14}C was omitted, an essentially similar elution profile was obtained, indicating that trace amounts of radioactive hypoxanthine did not alter the elution pattern. When 0.1 mM 2,4-dinitrophenol was included in another control experiment, no ultraviolet-absorbing material was present in the area normally occupied by ADP (tubes 40–45) and ATP (tubes 60–65). A slightly larger amount of radioactive ultraviolet-absorbing substance, probably AMP, was found in fraction B.

When 6-methylamino-9-(3′-deoxy-β-D-ribofuranosyl)purine was incubated under the same conditions, the elution pattern obtained from the acid-soluble fraction was generally similar to that of the control experiment except for a conspicuous ultraviolet-absorbing peak at C (expt 4). This nonradioactive material appeared to be the monophosphate of the analog by absorption spectroscopy and phosphate analysis (Table V). Fraction E, although not characterized, appeared to be similar to the material found in the corresponding zone in the control experiment (expt 3). This experiment was repeated with uniformly tritiated 6-methylamino-9-(3′-deoxy-β-D-ribofuranosyl)purine and the elution pattern is shown in expt 5. The tritiated substance in fraction C again appeared to be the monophosphate of 6-*N*-methylated congener. These experiments showed that nucleosides containing a methylamino group on carbon 6 were not metabolized further to the 5′-di- and 5′-triphosphates. It should be noted that the ultraviolet absorption patterns between zones D and G in expt 4 and 5 were similar to that of the control experiment (expt 3).

The elution profiles obtained from cells incubated with 6-dimethylamino-9-(3′-deoxy-β-D-ribofuranosyl)-purine, 6-ethylamino-9-(3′-deoxy-β-D-ribofuranosyl)-purine, and 2,6-diamino-9-(3′-deoxy-β-D-ribofuranosyl)-purine were essentially similar to each other (expt 6, 7, and 9) and to the control experiment (expt 3). There was no evidence of phosphorylation of these analogs.

Because 6-methylamino-9-(3′-deoxy-β-D-ribofuranosyl)purine was phosphorylated to the monophosphate but not to the di- and triphosphate level, the metabolism of an analogous compound, 6-methylamino-9-(β-D-ribofuranosyl)purine, was examined (expt 8). This nucleoside containing a ribose instead of a 3-deoxyribose moiety was also converted to the monophosphate (fraction C) but not metabolized further to the di- and triphosphates.

As the nucleosides, aside from 3′-deoxyadenosine, were not phosphorylated to the 5′-triphosphate level, experiments were performed to see if the phosphorylation of 3′-deoxyadenosine could be affected by coincubation with either adenosine or with 6-methylamino-9-(3′-deoxy-β-D-ribofuranosyl)purine. For this purpose, 10 mg of 3′-deoxyadenosine was incubated with either 10 mg of adenosine or 10 mg of the methylated congener. As shown in expt 10, 6-methylamino-9-(3′-deoxy-β-D-ribofuranosyl)purine did not significantly alter the phosphorylation of 3′-deoxyadenosine. Fractions C, D, and F were found to be 3′-deoxyadenosine 5′-mono-, 5′-di-, and 5′-triphosphate. On the other hand, adenosine completely suppressed the phosphorylation of 3′-deoxyadenosine. The elution pattern from this experiment (not shown in Figure 2) was similar to that of expt 2 in which adenosine alone was used. The latter results suggested that adenosine competed with 3′-deoxyadenosine for phosphorylation.

The possible formation of phosphorylated derivatives of 3′-amino-3′-deoxyadenosine by whole ascites cells was next investigated. Three prominent ultraviolet-absorbing zones designated C, D, and F were obtained. The elution profile (not shown here) was similar to that obtained with 3′-deoxyadenosine (Figure 2, expt 1). These fractions showed absorption spectra similar to

[1] Abbreviations used: AMP, adenosine monophosphate; ADP, adenosine diphosphate; ATP, adenosine triphosphate; NAD, nicotinamide–adenine dinucleotide; NADP, NAD phosphate; GTP, guanosine triphosphate; 3′-dATP, 3′-deoxyadenosine 5′-triphosphate; CTP, cytidine triphosphate; UTP, uridine triphosphate.

TABLE VI: Properties of Nucleosides and Fractions Obtained by Dowex-1 Formate Chromatography.[a]

Nucleoside or Fractions	λ_{min} (mμ)	λ_{max} (mμ)	ϵ_{max}	250/260	280/260	Total P/ Base	Acid Labile P/ Base
3′-Amino-3′-deoxyadenosine	227	259	14,300	0.79	0.15		
Fraction C	229	258		0.81	0.17	1.13	0
Fraction D	227	258		0.82	0.19	1.97	1.03
Fraction F	228	259		0.81	0.16	3.05	1.97
Puromycin aminonucleoside	237	275	16,900	0.45	1.50		

[a] The spectra were taken at pH 7.

TABLE VII: The Effects of 3′-NH_2-3′-dATP on the Incorporation of Ribonucleotide Triphosphate into RNA Catalyzed by RNA Polymerase from *Micrococcus lysodeikticus*.[a]

	Mμmoles of Labeled Nucleotide Triphosphate Incorpd							
Incubation System	UTP-2-^{14}C	% Inhib	ATP-8-^{14}C	% Inhib	GTP-8-^{14}C	% Inhib	CTP-2-^{14}C	% Inhib
Complete system	3.19							
+ 50 mμmoles of 3′-NH_2-3′-dADP	3.16	1						
+ 20 mμmoles of 3′-NH_2-3′-dATP	0.65	80						
+ 50 mμmoles of 3′-NH_2-3′-dATP	0.52	84						
Complete system			4.23					
+ 20 mμmoles of 3′-NH_2-3′-dATP			0.85	80				
+ 50 mμmoles of 3′-NH_2-3′-dATP			0.51	88				
Complete system					4.24			
+ 20 mμmoles of 3′-NH_2-3′-dATP					0.98	77		
+ 50 mμmoles of 3′-NH_2-3′-dATP					0.59	86		
Complete system							3.46	
+ 50 mμmoles of 3′-NH_2-3′-dADP							3.50	0
+ 20 mμmoles of 3′-NH_2-3′-dATP							0.60	81
+ 50 mμmoles of 3′-NH_2-3′-dATP							0.40	89

[a] Complete system in a volume of 0.50 ml contained 50 mμmoles each of UTP, ATP, GTP, and CTP (labeled or unlabeled), 50 μmoles of Tris buffer (pH 7.6), 0.5 μmole of spermidine phosphate, 1 μmole of $MnCl_2$, 60 μg of calf-thymus DNA, and enzyme preparation (50 μg of protein). After incubation for 10 min at 30°, the radioactivity in the acid-insoluble fractions was determined.

that of 3′-amino-3′-deoxyadenosine (Table VI). The molecular extinctive coefficient of the nucleoside was determined to be 14,300. Using this ε value for the nucleotides, fractions C, D, and F were found to contain 1.13, 1.97, and 3.05 μmoles of phosphate/μmole of nucleoside as measured by the Fiske–Subbarow method (1925). After treatment in 1 N HCl for 10 min at 100°, the μmoles of acid-labile phosphate/μmole of nucleoside for fractions C, D, and F were found to be 0, 1.03, and 1.97, respectively. Approximately 10 μg of fractions D and F were subjected to paper electrophoresis in 0.05 M citrate buffer (pH 3.5) at 20 v/cm for 3.5 hr. Fractions D and F migrated considerably slower than ADP and ATP, respectively. For comparative purposes, 3′-deoxyadenosine 5′-triphosphate (3′-dATP) obtained in a previous experiment (Shigeura and Gordon, 1965) was also subjected to electrophoresis. ATP and 3′-dATP displayed the same electrophoretic mobility. Fraction A (1 μmole) was found to consume 0.96 μmole of periodate when tested by the spectrophotometric method (Dixon and Lipkin, 1954). Fractions C, D, and F were, therefore, characterized as 3′-amino-3′-deoxyadenosine 5′-mono-, 5′-di- and 5′-triphosphate, respectively. In this experiment, the micromoles of 5′-mono-, 5′-di-, and 5′-triphosphate formed were 1.25, 1.93, and 3.18, respectively. The phosphorylated derivatives are hereafter abbreviated as 3′-NH_2-3′-dAMP, 3′-NH_2-3′-dADP, and 3′-NH_2-3′dATP, respectively.

When 6-dimethyl-3′-amino-3′-deoxyadenosine was used, the elution profile obtained was similar to that of

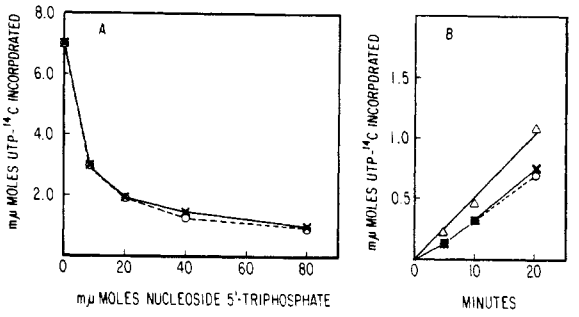

FIGURE 3: Effects of 3′-dATP and 3′-NH_2-3′-dATP on UTP-2-^{14}C incorporation and inhibition of RNA synthesis. (A) Comparative effects of various concentrations of 3′-dATP (solid line) and 3′-NH_2-3′-dATP (broken line) on UTP-2-^{14}C incorporation into RNA. Complete system in a volume of 0.50 ml contained 100 mμmoles each of ATP, GTP, CTP, and UTP-2-^{14}C (18,800 cpm), 50 μmoles of Tris buffer (pH 7.6), 0.5 μmole of spermidine phosphate, 1 μmole of $MnCl_2$, 60 μg of calf-thymus DNA, and enzyme preparation (50 μg of protein); incubated at 30° for 10 min. (B) Kinetics of inhibition of RNA synthesis by 3′-dATP (×—×) and 3′-NH_2-3′-dATP (⊙—⊙). Control experiment without nucleotide 5′-triphosphate (Δ—Δ). Complete system in a volume of 1.50 ml contained 300 mμmoles of ATP, GTP, CTP, and UTP (47,000 cpm), 150 μmoles of Tris buffer (pH 7.6), 1.5 μmoles of spermidine phosphate, 3 μmoles of $MnCl_2$, 180 μg of calf-thymus DNA, and enzyme preparation (150 μg of protein); incubated at 30° and 0.40-ml aliquots were removed at indicated intervals, purified, and measured for radioactivity.

the control experiment (Figure 2, expt 3). The results indicated that under these conditions puromycin aminonucleoside was not phosphorylated by Ehrlich ascites cells.

Experiments with RNA Polymerase. Since 3′-deoxyadenosine 5′-triphosphate was previously found to exert a pronounced inhibitory effect on the activity of RNA polymerase from *M. lysodeikticus* (Shigeura and Gordon, 1965), similar studies were done with 3′-NH_2-3′-dATP. As shown in Table VII, 3′-NH_2-3′-dATP markedly suppressed the incorporation of each of the labeled ribonucleotide triphosphates into RNA catalyzed by DNA-dependent RNA polymerase. When the ratio of concentration of ATP to 3′-NH_2-3′-dATP was 2.5, the per cent inhibition was 77–81. 3′-NH_2-3′-dADP had no effect on the system.

The activity of 3′-NH_2-3′-dATP shown above resembled that of 3′-dATP (Shigeura and Gordon, 1965) which was found to be incorporated into RNA by RNA polymerase from *M. lysodeikticus* (Shigeura and Boxer, 1964). Results were obtained which indicated that 3′-dATP was incorporated in lieu of ATP into a growing polynucleotide chain, and it was suggested that the absence of a hydroxyl group on carbon 3 of the deoxyribose moiety prevented further elongation of that nascent RNA chain. Because 3′-NH_2-3′-dATP appeared to function as 3′-dATP, subsequent experiments with 3′-NH_2-3′-dATP were performed in conjunction with 3′-dATP.

The effects of the two structurally related triphosphates on DNA-dependent syntheses of polyadenylate and polyuridylate are shown in Table VIII. Poly-A formation was strongly inhibited by both triphosphates whereas poly-U synthesis was essentially unaffected. It should be noted that these results with 3′-dATP have already been reported (Shigeura and Gordon, 1965) and were repeated here merely for comparative purposes.

TABLE VIII: Comparative Effects of 3′-NH_2-3′-dATP and 3′-dATP in DNA-Dependent Poly A and Poly U Formation by RNA Polymerase.[a]

Incubation System	Mμmoles Ribonucleotide Triphosphate Incorpd			
	ATP-8-^{14}C	% Inhib	UTP-2-^{14}C	% Inhib
Complete system	7.05			
+ 8 mμmoles of 3′-NH_2-3′-dATP	0.69	90		
+ 8 mμmoles of 3′-dATP	0.60	91		
Complete system			0.67	
+ 4 mμmoles of 3′-NH_2-3′-dATP			0.61	9
+ 8 mμmoles of 3′-NH_2-3′-dATP			0.60	11
+ 20 mμmoles of 3′-NH_2-3′-dATP			0.63	6
+ 4 mμmoles of 3′-dATP			0.74	0
+ 8 mμmoles of 3′-dATP			0.59	12
+ 20 mμmoles of 3′-dATP			0.60	11

[a] Complete system in a volume of 0.50 ml contained 100 mμmoles each of ATP or UTP (18,800 cpm), 50 μmoles of Tris buffer (pH 7.6), 0.5 μmole of spermidine phosphate, 1 μmole of $MnCl_2$, 60 μg of calf-thymus DNA, and enzyme preparation (50 μg of protein).

The effects of various concentrations of 3′-NH_2-3′-dATP and 3′-dATP on UTP-2-^{14}C incorporation into RNA catalyzed by DNA-dependent RNA polymerase are shown in Figure 3A. The inhibitory activities of these two analogs of ATP on this reaction system were essentially identical.

If one may assume at this point that 3′-NH_2-3′-dATP was functioning as 3′-dATP in being incorporated into RNA and thus preventing further RNA chain elongation, addition of increasing amounts of 3′-NH_2-3′-

dATP to a system lacking ATP should not stimulate the incorporation of any one of the three remaining ribonucleotide triphosphates. As shown in Table IX, this appeared to be the situation. In the absence of ATP, addition of increasing amounts of either 3′-NH_2-3′-dATP or 3′-dATP did not significantly stimulate the incorporation of GTP-8-^{14}C into RNA.

TABLE IX: Lack of Stimulation of GTP-8-^{14}C Incorporation into RNA by 3′-NH_2-3′-dATP or 3′-dATP.[a]

Incubation System	mμmoles of GTP-8-^{14}C Incorpd
Complete system	5.61
− ATP	0.11
− ATP + 8 mμmoles of 3′-NH_2-3′-dATP	0.16
− ATP + 20 mμmoles of 3′-NH_2-3′-dATP	0.06
− ATP + 50 mμmoles of 3′-NH_2-3′-dATP	0.06
− ATP + 8 mμmoles of 3′-dATP	0.08
− ATP + 20 mμmoles of 3′-dATP	0.06
− ATP + 50 mμmoles of 3′-dATP	0.11
Complete system − $MnCl_2$	0.03
Complete system − spermidine phosphate	4.91

[a] Complete system in a volume of 0.50 ml contained 50 mμmoles each of ATP, UTP, CTP, and GTP-8-^{14}C (18,800 cpm), 50 μmoles of Tris buffer (pH 7.6), 0.5 μmole of spermidine phosphate, 1 μmole of $MnCl_2$, 60 μg of calf-thymus DNA, and enzyme preparation (50 μg of protein). Incubated at 30° for 10 min.

A study of the kinetics of inhibition of RNA synthesis by the two structurally related analogs, shown in Figure 3B, also failed to demonstrate major differences in the activities of these two compounds.

The remarkable resemblance of the effects of 3′-NH_2-3′-dATP to those of 3′-dATP on various activities of RNA polymerase from *M. lysodeikticus* suggested that the mechanism of action of these two analogs of ATP was essentially similar. This would imply that 3′-NH_2-3′-dATP was also incorporated into RNA, thereby preventing further elongation of the nascent polynucleotide. Such a situation would be expected to prevail if the amino group on the ribose carbon 3 were unable to form a stable covalent bond with the next incoming ribonucleotide triphosphate during RNA synthesis. A question may be raised about the possibility that a labile phosphoamide bond was formed but subsequently cleaved when the incubation was terminated by the addition of cold 5% trichloroacetic acid. The latter event, although possible, appeared to be unlikely because of the striking similarity in the kinetics of inhibition shown by the two analogs, in addition to the similar, if not identical, inhibitory effects observed on polynucleotide synthesis primed by either DNA or synthetic homopolymer.

Discussion

Studies from various laboratories have demonstrated that antimetabolites of adenine, guanine, and their nucleosides are usually converted to the nucleotides which are then able to exert their influences on various biological processes (Brockman, 1963; Emmelot, 1964). As 3′-deoxyadenosine was resistant to phosphorylase action (LePage and Junga, 1965) (see also Table IV), the mechanism of its conversion to 3′-deoxyadenosine 5′-monophosphate proceeded *via* nucleoside kinase. Results described in this paper have shown that the congeners of 3′-deoxyadenosine were also not cleaved by ascites cells. The experiments described here were, therefore, essentially a study of the effects of certain structural modifications of 3′-deoxyadenosine on the activity of adenosine nucleoside and nucleotide kinases, and, subsequently, on the synthesis of nucleic acids. The possibility that the observed effects described in this report were due to differences in the permeability of the various nucleosides cannot be completely excluded; however, Duggan and Titus (1962) have shown that exogenous 6-methylaminopurine was utilized by Ehrlich ascites cells. In the following discussion, it is assumed that permeability of the nucleosides was not a controlling factor in the experiments.

Since 3′-deoxyadenosine was readily phosphorylated by ascites cells, the results reported here demonstrated that removal of the 6-amino moiety or substitution of one or both hydrogen atoms on 6-amino by an alkyl group drastically interfered with phosphorylation in the 5′ position of the sugar. The presence of a monomethyl group at this position permitted the formation of only a small amount of mononucleotide. The latter result suggested that di- and triphosphate kinases were inhibited by the nucleoside or nucleoside monophosphate, or 6-methylamino-9-(3′-deoxy-β-D-ribofuranosyl)purine 5′-monophosphate could not serve as substrate for myokinase. It should be noted that a large amount of ATP was present in this system. It is also interesting to note that this stringent structural specificity for purine nucleoside phosphorylation also applied to a member of the ribose series, 6-methylamino-9-(β-D-ribofuranosyl)-purine 5′-monophosphate, indicating that a change in structure on carbon 3 of the pentose moiety did not affect this particular process.

Thus, the experiments reported here indicated that the 6-amino moiety was essential for extensive phosphorylation of adenosine analogs by Ehrlich ascites cells. The recent demonstration that 2-fluoroadenosine was readily phosphorylated to 5′-triphosphate by Ehrlich ascites cells (Shigeura *et al.*, 1965), and the results reported here on the inability of puromycin aminonucleoside to be phosphorylated in contrast to the ease with which 3′-amino-3′-deoxyadenosine was phosphorylated are consistent with the above hypothesis. 7-Deazaadenosine (tubercidin) (Acs *et al.*, 1964), arabinosyladenine (Brink and LePage, 1964), and xylosyladenine

(Ellis and Le Page,1965), other adenosine analogs with free 6-amino groups, have also been found to be phosphorylated to the triphosphate level in different biological systems. It should be emphasized, however, that the mere presence of a 6-amino group does not necessarily guarantee the biological conversion of the nucleoside to the 5′-triphosphate. For example, no evidence has yet been obtained for the formation of 5′-triphosphates of psicofuranine (Magee and Eberts, 1961) or 8-azaadenine (Bergquist and Matthews, 1962). The inability of 2,6-diamino-9-(3′-deoxy-β-D-ribofuranosyl)purine to be phosphorylated may be due to the possibility that this nucleoside is an analog of guanosine. Guanosine kinase activity has not yet been demonstrated (Utter, 1960).

The results of these experiments also showed that phosphorylation and the extent of phosphorylation were directly correlated with the effectiveness of the nucleosides as inhibitors of 5-phosphoribosylamine synthesis and of hypoxanthine incorporation into nucleic acids in whole ascites cells (Tables I–III). It is noteworthy that 3′-deoxyadenosine, 3′-amino-3′-deoxyadenosine, and 2-fluoroadenosine, nucleosides shown to be phosphorylated to the 5′-triphosphate level, were the most efficient inhibitors. 6-Methylamino-9-(3′-deoxy-β-D-ribofuranosyl)purine and 6-methylamino-9-(β-D-ribofuranosyl)purine, presumably in the form of 5′-monophosphates, were somewhat less active. The remaining analogs that were not phosphorylated at all showed only minimal activities in both test systems. It may, therefore, be concluded that the lack of activity of the latter group of nucleosides can be attributed to the complete absence of phosphorylation.

A variety of methylated bases have now been found to be present in nucleic acids (Hall, 1965) and recent studies have shown that methylation of some of these bases occurred at the polynucleotide level (Mandel and Borek, 1961; Biswas *et al.*, 1961; Gold *et al.*, 1963). Such a unique pathway for the formation of methylated constituents of nucleic acids apparently renders the biosynthesis of methylated nucleotides unnecessary. Indeed, alkylated nucleotide 5′-triphosphates have not as yet been found in the acid-soluble fractions of mammalian cells. The results reported here demonstrating the inability of various N^6-alkylated nucleosides to be phosphorylated to the triphosphate level and consequently their inability to serve as nucleic acid precursors are consistent with the above findings.

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