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Adenosine Kinase of Sarcoma 180 Cells

N^6-Substituted Adenosines as Substrates and Inhibitors

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SUMMARY

Adenosine kinase (ATP:adenosine 5′-phosphotransferase, EC 2.7.1.20) was partially purified by DEAE-cellulose column chromatography from Sarcoma 180 cells grown *in vitro*. This enzyme preparation, which was free of adenosine deaminase (adenosine aminohydrolase, EC 3.5.4.4) but contained adenylate kinase (ATP:AMP phosphotransferase, EC 2.7.4.3), was studied with respect to its kinetic properties and specificity for substrates and inhibitors.

At pH 7.0 and 35° the K_m for adenosine was 0.5 μM and the V_{max} was 63 mμmoles/mg of protein per minute. Strong substrate inhibition was observed at adenosine concentrations greater than 4 μM, 50% inhibition occurring at about 100 μM. The reaction required ATP (K_m = 200 μM) and Mg^{++}. The optimal Mg^{++} concentrations were 0.1 and 0.25 mM at 0.5 and 2.5 mM ATP, respectively. Concentrations of Mg^{++} higher than these were inhibitory, and Mn^{++} substituted poorly for Mg^{++}.

Most of the N^6-substituted adenosine analogues which were substrates of adenosine kinase inhibited the growth of S-180 cells *in vitro*. Among these were two compounds which are also known to be potent cytokinins in plant systems, namely, N^6-furfuryl- and N^6-(Δ^2-isopentenyl)adenosine. The 5′-monophosphate of the latter compound was not phosphorylated further by adenylate kinase. The N^6-substituted adenosines which were poorly or not at all phosphorylated by adenosine kinase were also poor inhibitors of S-180 cells *in vitro*. Several of these were potent inhibitors of the kinase, such as N^6-phenyladenosine, which had a K_i value of 0.6 μM.

INTRODUCTION

Adenosine kinase (ATP:adenosine 5′-phosphotransferase, EC 2.7.1.20) is an enzyme with broad substrate specificity involved in the biological activation of several cytotoxic purine nucleosides (1). It is also implicated in adenosine transport into chick fibroblast cells (2), whereas in mouse Sarcoma 180 cells the membrane transport of adenosine appears to be unrelated to the functions of adenosine kinase (3, 4). The enzyme was first demonstrated in yeast and in mammalian tissues (5), and it has been partially purified from rabbit liver (6), from human tumor cells grown *in vitro* (7), and, most recently, from brewers' yeast (8). The study of adenosine kinase in crude cell extracts using adenosine as the substrate is difficult because of interference by adenosine deaminase. This paper describes the partial

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purification and some properties of adenosine kinase of Sarcoma 180 cells grown *in vitro*. The kinetic characteristics and substrate and inhibitor specificity, especially as concerns N^6-substituted adenosine analogues, are also described.

MATERIALS AND METHODS

Compounds. Compounds used in the present study were purchased from the following sources: adenosine-8-^{14}C and ATP-γ-^{32}P, from Amersham/Searle Corporation, Arlington Heights, Ill.; cytidine-2-^{14}C and guanosine-8-^{14}C, from Schwarz-Mann, Orangeburg, N. Y.; inosine-8-^{14}C and AICAR,[1] from Calbiochem; uridine-2-^{14}C, from New England Nuclear Corporation, Boston; N^6-furfuryladenosine (kinetin riboside), from K and K Laboratories; rabbit muscle adenylate kinase (Grade III) and 5′-nucleotidase of *Crotalus adamanteus* venom (grade II), from Sigma Chemical Company; microgranular DEAE-cellulose (Whatman DE 52) from Reeve Angel, Clifton, N. J. Toyocamycin was obtained through the courtesy of Dr. George Acs, Institute of Muscle Disease, Inc., New York. N^6-(*cis*-2-Chlorobuten-2-yl)adenosine and the corresponding *trans* derivative were obtained through the courtesy of Dr. Sidney Hecht, University of Wisconsin; ^{35}S-labeled 6-methylmercapto-9-(β-D-ribofuranosyl)purine was obtained through the courtesy of Dr. D. H. W. Ho, M. D. Anderson Tumor Institute, Houston, Tex.; *p*-nitrobenzylthioguanosine was a gift from Dr. A. R. P. Paterson, University of Alberta, Edmonton. N^6-Methyl-, -isoamyl-, -pentyl-, -hexyl-, -allyl-, -phenyl-, -benzyl-, -2-ethoxyethyl-, and -phenoxyethyladenosine and N^6-(Δ^2-isopentenyl)tubercidin were synthesized and kindly supplied by Dr. M. H. Fleysher of this department. N^6-(Δ^2-Isopentenyl)adenosine was a product of Stark Associates, Buffalo, N. Y., recrystallized from ethanol by Dr. M. H. Fleysher and found to be more than 99% pure.

Cell extract. The parent mouse Sarcoma 180 (S-180) cell line of Foley and Drolet (9), maintained for 15 years in this laboratory, was used in the present study. The cells for enzyme isolation were grown in Roux flasks in monolayer culture in Eagle's medium (10) supplemented with 5% horse serum. One day after the last medium change, the cells were scraped off with a rubber policeman, collected in a small volume of the medium, and centrifuged at 1000 rpm for 5 min. After weighing, the cells were resuspended in 2 volumes of isotonic salt solution, centrifuged at 2000 rpm for 10 min, weighed, and stored at −70°. The frozen cells (2-g pellet) were suspended in 2 ml of 5 mM Tris-chloride buffer, pH 7.0, containing 1 mM EDTA and 5 mM NaCl (buffer A). The suspension was homogenized in a Potter-Elvehjem homogenizer until most of the cells were broken as determined microscopically. This required no more than 20 up-down strokes. All operations were carried out at or below 5°. The homogenate was diluted to 6 ml with buffer A, mixed, and centrifuged at 105,000 × *g* for 60 min, using a Beckman model L2-65B ultracentrifuge. The supernatant fluid was dialyzed against 1.5 liters of buffer A for 16 hr, and the dialysate was centrifuged at 105,000 × *g* for 30 min to remove the precipitate which formed.

Adenosine kinase assay. The assay was based on the conversion of adenosine-8-^{14}C to nucleotides and their estimation after chromatographic separation from the remaining substrate. Unless otherwise specified, the reaction mixture (0.4 ml) contained 0.1 mM adenosine (2.5 μCi/μmole) 2.5 mM ATP, 0.25 mM $MgCl_2$, 50 mM potassium phosphate buffer (pH 7.0), and the enzyme. The reaction was started by adding the enzyme (20–160 μl) to the assay mixture, which had been warmed to 35°. The reaction, at 35°, was stopped by immersing the assay tubes in a boiling water bath for 2 min. An aliquot (50 μl) was subjected to descending chromatography for 4 hr on Whatman No. 3MM paper (2.5 × 35 cm) with 1-butanol–acetic acid–water (20:3:7) as the solvent (solvent A) (11). In this solvent system all of the nucleotides remain at the origin, while adenosine is clearly separated. The paper strip was cut into 1-cm sections, which were counted for ^{14}C content in 10 ml of scintillator solution consisting of 5 g of 2,5-diphenylox-

[1] The abbreviation used is: AICAR, 1-β-D-ribofuranosyl-5-amino-4-imidazolecarboxamide.

azole (PPO) and 0.3 g of 1,4-bis[2-(5-phenyloxazolyl)]benzene (POPOP) in 1 liter of toluene in a Packard Tri-Carb liquid scintillation counter. The counting efficiency was 65%. The control consisted of the complete reaction mixture immersed in boiling water bath immediately after addition of the enzyme. Experimental counts were always corrected using the corresponding controls.

When separation of AMP, ADP, and ATP was necessary, the chromatography was carried out with solvent B [isobutyric acid–water–NH_4OH (66:33:1)] for 16 hr.

The assay for formation of nucleoside phosphates of unlabeled adenosine analogues was carried out as described above, except that ATP-γ-^{32}P (1 μCi/μmole) replaced ATP. Each assay mixture contained 12.8 μg of protein, and the incubation was conducted for 30 min. Solvent B was used for chromatography; the strip was counted for ^{32}P content as described above. The counting efficiency was 60%.

Testing of adenosine kinase inhibitors. Inhibitory activity was tested initially by incubating for 2 min a mixture of 0.82 μM adenosine, 1.8 μg of protein, 2.5 mM ATP, 0.25 mM $MgCl_2$, and buffer as detailed above, together with 75 or 150 μM inhibitor. In the inhibitor-free control the conversion of adenosine to nucleotides was about 50%. K_i values for the inhibitors were determined at two concentrations of the inhibitor and five concentrations of adenosine (0.55–1.64 μM). The inhibitor-free controls were incubated for 2 min, and the tubes containing inhibitor, for 5 min. The K_i value was the average of the two values obtained from the Lineweaver-Burk plots for competitive inhibitors (12).

Adenosine deaminase assay. The deaminase activity was measured spectrophotometrically by the method of Kalckar (13). The reaction mixture contained 50 mM potassium phosphate buffer, pH 7.0, and an appropriate amount of enzyme in a total volume of 3.2 ml. The linear decrease in absorption at 265 mμ at 37° for the first 5 min was measured in a Zeiss PMQ II spectrophotometer equipped with a thermostated compartment. The enzyme activity was calculated according to Kalckar.

Testing for growth inhibition. Monolayer cultures of S-180 cells in T-15 flasks were used. In 2 ml of medium (10), 200,000 cells were inoculated per T-15 flask. After 24 hr the medium was replaced with one containing the inhibitor, and growth was allowed to proceed for 6 or 7 days with two or three more changes of medium. The growth was estimated by protein assay (14). The inhibitors were applied at several different concentrations, each tested in triplicate.

Protein determination. Protein was determined according to the method of Lowry *et al.* (14), with bovine serum albumin as the standard.

RESULTS

Partial purification of adenosine kinase. The S-180 cell extract was subjected to DEAE-cellulose column chromatography using a linear NaCl gradient (see Fig. 1). Adenosine kinase was generally eluted between fractions 52 and 68, closely followed by adenosine deaminase, with only minor overlapping. The fractions containing adenosine kinase but no deaminase were pooled. The degree of purification was approximately 12-fold when compared to the crude cell extract. The preparation contained no significant apyrase, adenylate deaminase, or nucleoside phosphorylase activity that might have interfered in the adenosine kinase assay, although it contained adenylate kinase.

Preliminary experiments revealed a lack of linearity between the rate of phosphorylation and the enzyme concentration, suggesting inactivation of the enzyme at high dilutions. Linearity was achieved when crystalline bovine serum albumin was added to the assay mixture. Bovine serum albumin was therefore routinely added (500 μg/ml) to the pooled fractions, to give a minimum concentration of 10 μg of albumin per assay tube. Under these conditions, when stored in the refrigerator, the enzyme preparation lost only 10% of its kinase activity in 34 days, while at −70° no loss of activity was observed after 2 months.

The elution pattern of adenosine kinase activity when adenosine-8-^{14}C was used as the substrate coincided with the pattern for

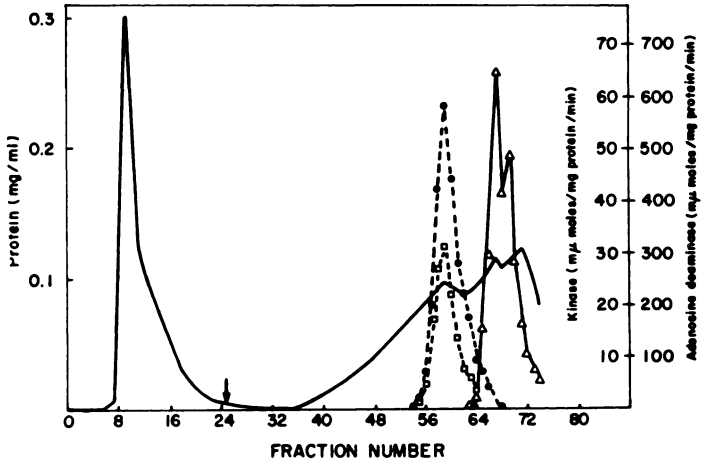

FIG. 1. *Chromatography of adenosine kinase on DEAE-cellulose*

S-180 cell extract in buffer A (3 ml, 30 mg of protein) was applied to a column of DEAE-cellulose (2 × 20 cm) that had been equilibrated with buffer A. The column then was washed with 75 ml of buffer A, and a linear NaCl gradient was applied at the point indicated by the arrow. This consisted of two communicating, identical vessels of buffer A and buffer A containing 500 mM NaCl. The flow rate was 1 ml/min; 3-ml fractions were collected. ●---●, adenosine kinase activity; □---□, 6-methylmercaptopurine ribonucleoside-^{35}S kinase activity. The relatively low activity for methylmercaptopurine ribonucleoside as compared with the activity for adenosine is most probably (see Table 1) due to 6-day storage of the fractions in a refrigerator without albumin before they were assayed with methylmercaptopurine ribonucleoside. △——△, adenosine deaminase activity; ——, protein.

6-methylmercapto-9-(β-D-ribofuranosyl)purine-^{35}S as a substrate (Fig. 1). This suggests that the same enzyme catalyzes the phosphorylation of the two compounds, as has been observed also by Schnebli *et al.* (7).

General properties of adenosine kinase. The linearity of adenosine phosphorylation with respect to time and enzyme concentration is shown in Fig. 2. Analysis of the reaction products at 30 min showed formation of 45% AMP and 55% ADP, with a trace of ATP. The dependence of the reaction velocity on substrate concentration is presented as a semilogarithmic plot in Fig. 3, which shows that adenosine phosphorylation was maximal at approximately 4 μM. Higher concentrations of adenosine were inhibitory, 50% inhibition being observed at about 100 μM adenosine. The K_m and V_{max} values for adenosine were 0.5 μM and 63 mμmoles/mg of protein per minute, respectively (Fig. 3). This compared well with K_m values reported for the enzyme from Ehrlich ascites cells [2.8 μM (11)], H.Ep.2 cells [1.8 μM (7)], and rabbit liver [1.6 μM (6)].

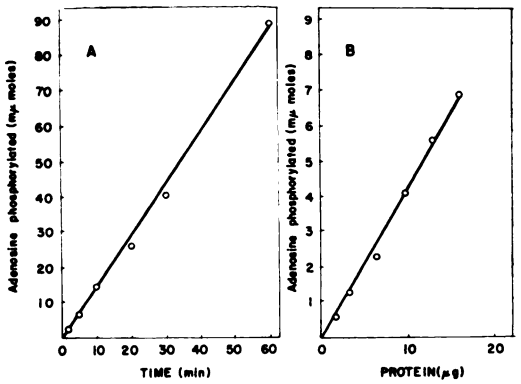

FIG. 2. *Phosphorylation of adenosine as a function of time (A) and protein concentration (B)*

A. Experimental conditions were as described under MATERIALS AND METHODS, except that the volume of the solution was 1 ml and it contained 44 μg of protein. Aliquots of 100 μl were withdrawn at specified time intervals and analyzed by chromatography with solvent A.

B. Adenosine phosphorylation was measured over 15 min as a function of protein concentration. Experimental conditions were as described under MATERIALS AND METHODS.

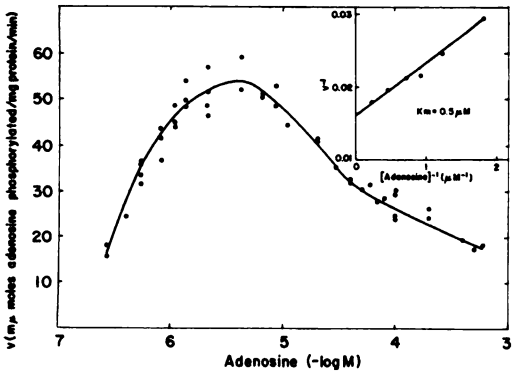

FIG. 3. *Phosphorylation of adenosine as a function of substrate concentration*

Experimental conditions were as described under MATERIALS AND METHODS. At 0.27–8.7 μM adenosine-8-^{14}C (specific activity, 47 μCi/μmole), 1.8 μg of protein were used per assay tube. The time of incubation was 2 min at 0.27–1.4 μM adenosine and 5 min at 2.2–8.7 μM adenosine. At 10–500 μM adenosine (specific activity, 1 μCi/μmole), 8.8 μg of protein were used, and the time of incubation was 15 min. A Lineweaver-Burk plot (12) of these data, based on average velocity values, is shown in the inset.

Requirement for ATP. The phosphorylation of adenosine was dependent on the presence of ATP (Fig. 4). The K_m value for ATP was 220 μM (Fig. 4), and high concentrations of ATP, such as 7.5 mM, were not significantly inhibitory at 100 μM adenosine. At the optimal adenosine concentration (4.4 μM), the K_m for ATP was 180 μM. The nucleotide triphosphate specificity for the reaction was not investigated here, but ITP and GTP are also known to act as phosphate donors (7).

Mg^{++} requirement. Figure 5 shows the Mg^{++} requirements of the adenosine kinase reaction at two concentrations of ATP. At 2.5 mM ATP the optimum Mg^{++} concentration was 0.25 mM, while at 0.5 mM ATP it was 0.1 mM. Thus, the optimal ratio of Mg^{++} to ATP (0.1 and 0.2) differs substantially from unity. Furthermore, higher than optimal Mg^{++} concentrations were strongly inhibitory, in agreement with Murray (11), even though the Mg^{++}:ATP ratio was still below 1.0. The Mg^{++} requirement of this enzyme appears to be independent of ATP and of the formation of an Mg^{++}-ATP complex. Mn^{++} substituted poorly for Mg^{++}; at 0.12–1 mM it promoted less than 10% of the optimal reaction with Mg^{++}.

pH optimum. The pH optimum of adenosine kinase was at or below pH 5.5 in phos-

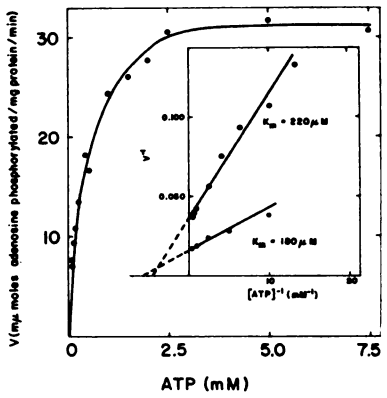

FIG. 4. *Phosphorylation of adenosine as a function of ATP concentration*

Experimental conditions were as described under MATERIALS AND METHODS, except that the ATP concentration was varied as indicated. Each assay tube contained 8.1 μg of protein, and the incubation time was 10 min. Lineweaver-Burk plots (12) of the same data (100 μM adenosine, ●——●) and of data obtained at 4.4 μM adenosine (○——○) are shown in the inset.

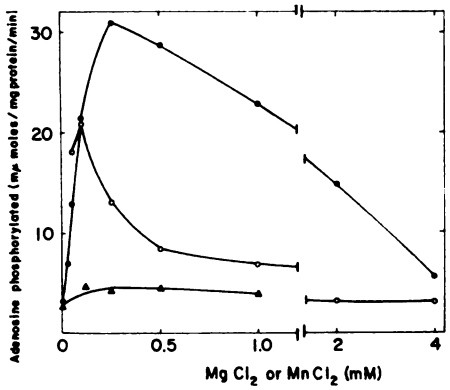

FIG. 5. *Effect of $MgCl_2$ and $MnCl_2$ on phosphorylation of adenosine*

For experimental conditions, see MATERIALS AND METHODS; 4 μg of protein were present in each assay tube, and the incubation time was 10 min. ●——● and ○——○, effect of $MgCl_2$ at 2.5 and 0.5 mM ATP, respectively; ▲——▲, effect of $MnCl_2$ at 2.5 mM ATP. The points represent averages of at least two experiments.

phate buffer (Fig. 6). This is similar to the pH optimum reported for the rabbit liver enzyme (6) but differs from that for Ehrlich ascites cells (11) and H.Ep.2 cells (7), for which a broad optimum of pH 6–7 has been reported.

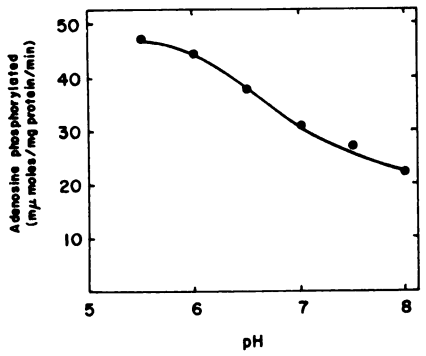

FIG. 6. *Effect of pH on phosphorylation of adenosine*

Experimental conditions were the same as described under MATERIALS AND METHODS. Each assay tube contained 4.4 μg of protein, and the incubation time was 10 min.

Substrate specificity. Table 1 lists the adenosine analogues found to be substrates of the adenosine kinase of S-180 cells. The unlabeled nucleosides (100 μM) were incubated with 2.5 mM ATP-γ-^{32}P (1 μCi/μmole) and 12.8 μg of protein for 30 min as described under MATERIALS AND METHODS. If the nucleoside was phosphorylated, two distinct radioactive peaks appeared on the chromatogram in solvent B. Inorganic phosphate and residual ATP had an R_F of about 0.2. This scattered peak probably contained polyphosphates of some of the analogues, since this enzyme preparation was contaminated by adenylate kinase. The small but distinct peak that moved farther from the origin than ATP but not as far as the unlabeled nucleoside was considered to represent nucleoside 5′-monophosphate. This assignment was based on the relative order of migration in this solvent system, which is

TABLE 1

Substrates of adenosine kinase

Adenosine analogue	^{32}P-Nucleotide formed[a]	Inhibition of 0.82 μM Adenosine-8-^{14}C phosphorylation		K_i	50% inhibition of growth of S-180 cells[b]
		At 75 μM	At 150 μM		
	mμmoles/mg protein/min	%	%	μM	μM
Adenosine	17.2[c]			(K_m 0.5)	
Tubercidin	62.2[c]		73	14.2	0.17
Toyocamycin	22.7[c]	100		0.6	0.05
N^6-Methyladenosine	48.4			18.0	>300
N^6-Furfuryladenosine	14.5			22.6	5.0
N^6-(Δ^2-Isopentenyl)adenosine	13.2			14.8	22.0
N^6-(*cis*-2-Chlorobuten-2-yl)adenosine	16.9		72	21.1	4.4
N^6-(*trans*-2-Chlorobuten-2-yl)adenosine	7.5		82	7.8	11.0
N^6-Benzyladenosine	5.1	68	82		>300
N^6-Allyladenosine	4.6	82	94		20
N^6-(Δ^2-Isopentenyl)tubercidin	3.1	79	92		30
6-Methylmercapto-9-(β-D-ribofuranosyl)purine	47.5		65	20.3	1.6

[a] Nucleosides were tested for substrate activity as described under MATERIALS AND METHODS, together with ATP-γ-^{32}P.

[b] During 6 or 7 days of growth; controls grew 15–20-fold.

[c] This figure represents only the monophosphate. The diphosphate which may also have formed is not included.

triphosphate < diphosphate < monophosphate < nucleoside.

Experimental evidence for the formation of 5′-monophosphate was obtained in the case of N^6-(Δ^2-isopentenyl)adenosine. The presumed monophosphate of the compound was eluted from the paper and digested with 1 unit of 5′-nucleotidase of *C. adamanteus* venom (Sigma type II) in 5 mM Tris-chloride buffer, pH 8.9, at 37° for 30 min. This treatment resulted in a total loss of ^{32}P label (see Fig. 7A), confirming that the compound indeed was 5′-monophosphate.

The initial velocity values given in Table 1 are based on the rate of formation of the corresponding nucleoside 5′-monophosphate. Thus, the rate of AMP formation from adenosine represents only about half the total reaction, since the remaining half of the substrate was known to be converted to ADP under these conditions. Also, for nucleosides such as tubercidin and toyocamycin, which are known to be converted to polyphosphates (15, 16), the actual velocity values are likely to be higher than those given in Table 1.

Adenosine kinase of S-180 cells did not catalyze the phosphorylation of inosine-8-^{14}C; guanosine-8-^{14}C, cytidine-2-^{14}C, uridine-2-^{14}C, or AICAR. These compounds also did not inhibit the phosphorylation of adenosine. AICAR has been shown to be phosphorylated by crude pigeon liver extracts (17). It appears, therefore, that a separate enzyme may be involved.

The relative rates of phosphorylation, as listed in Table 1, of 6-methylmercapto-9-(β-D-ribofuranosyl)purine, tubercidin, N^6-methyl- and N^6-allyladenosine, and toyocamycin compare well with reported values (6, 7). The phosphorylation of several N^6-substituted adenosines, not investigated before, is also reported here. Thus, some cytotoxic adenosine analogues which are also known to be potent cytokinins were substrates of this enzyme. Among these, N^6-furfuryladenosine (kinetin riboside) and N^6-(Δ^2-isopentenyl)adenosine were fairly good substrates. The K_m value for N^6-(Δ^2-isopentenyl)adenosine (11.4 μM) was determined (Fig. 7B). Recently it has been shown that crude extracts of mouse leukemia L-1210 cells converted N^6-(Δ^2-isopentenyl)-adenosine to its 5′-monophosphate (18). On the basis of the present results it seems that

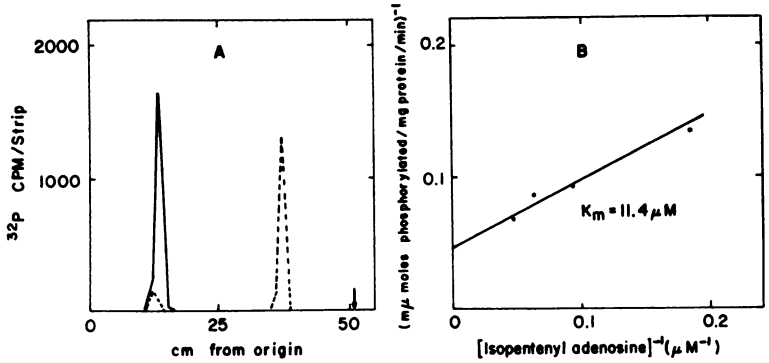

FIG. 7. *A. 5′-Nucleotidase treatment of ^{32}P-labeled N^6-(Δ^2-isopentenyl)adenosine phosphate*

Conditions are described under RESULTS. The dashed line represents the paper chromatogram with solvent B of N^6-(Δ^2-isopentenyl)adenosine phosphate incubated in the absence of 5′-nucleotidase (control), and the solid line, the chromatogram after incubation with the enzyme. The small peak on the dashed line, corresponding to inorganic phosphate, is presumably due to nonenzymatic degradation of isopentenyladenosine 5′-phosphate during incubation. The arrow indicates the solvent front.

B. Lineweaver-Burk plot for N^6-(Δ^2-isopentenyl)adenosine as a substrate of adenosine kinase

Experimental conditions were as described under MATERIALS AND METHODS, except that the concentration of ATP-γ-^{32}P (11.75 μCi/μmole) was 1 mM and the N^6-(Δ^2-isopentenyl)adenosine concentration was varied as indicated; 1.6 μg of protein was present in each assay tube, and the time of incubation was 5 min.

the enzyme involved in the conversion was adenosine kinase.

Inhibitors of adenosine kinase. All the substrates of adenosine kinase listed in Table 1 were also competitive inhibitors when adenosine-8-^{14}C was used as the substrate. Figure 8 presents evidence for this concerning N^6-furfuryl- and N^6-(Δ^2-isopentenyl)adenosine. The K_i for the latter compound (14.8 μM) agrees well with the K_m value, 11.4 μM, indicating that in this case K_m actually is the dissociation constant. Furthermore, it is clear that the N^6-substituted analogues occupy the same site on the kinase as the natural substrate, adenosine. Some of the N^6-substituted adenosine analogues, such as N^6-phenyl-, -pentyl-, and 2-phenoxyethyladenosine, which under the experimental conditions used could not be shown to be substrates, were found to be potent inhibitors of adenosine kinase (Table 2). N^6-Phenyladenosine was a competitive inhibitor, with a K_i 0.6 μM, almost equal to the K_m of adenosine.

5′-Monophosphate of N^6-(Δ^2-isopentenyl)-adenosine as a substrate of adenylate kinase. The possibility that N^6-(Δ^2-isopentenyl)-adenosine 5′-phosphate might be further phosphorylated by adenylate kinase was examined. For this purpose the ^{32}P-labeled monophosphate was prepared and separated from ATP-γ-^{32}P by paper chromatography. The eluted compound, at 2 μM, was incubated with 1 mM ATP, 1 mM $MgCl_2$, and 5 units of adenylate kinase in 50 mM potassium phosphate buffer, pH 7.0, for 20 min at 35°. No labeled di- or triphosphates were formed, as determined by chromatography with solvent B; instead, the original substrate was recovered unchanged. Under similar conditions AMP-8-^{14}C and ^{32}P-labeled tubercidin 5′-phosphate were almost completely converted to ADP, ATP, and tubercidin di- and triphosphates, respectively.

It is likely, therefore, that not only N^6-(Δ^2-isopentenyl)adenosine, but the other N^6-substituted adenosine analogues as well, were converted only to the monophosphate level within the cells. This has been reported to be the case for two compounds, N^6-methyladenosine and 6-methylmercapto-9-(β-D-ribofuranosyl)purine (19–21). In contrast, adenosine analogues without substitution at the 6-amino group, such as tubercidin, toyocamycin, and formycin A, are known to

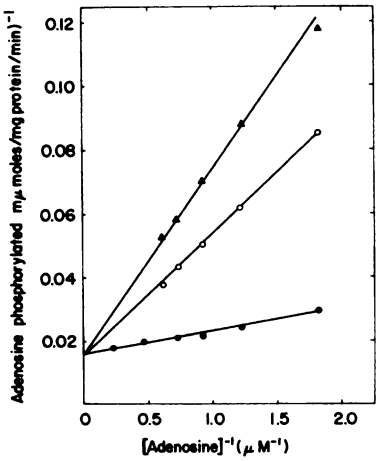

FIG. 8. *Competitive inhibition of adenosine phosphorylation*

Inhibitors: O——O, 54.5 μM N^6-furfuryladenosine; ▲——▲, N^6-(Δ^2-isopentenyl)adenosine; ●——●, no inhibitor. For experimental conditions, see MATERIALS AND METHODS.

TABLE 2

Inhibitors of adenosine kinase

These compounds were also tested as potential substrates in the presence of ATP-γ-^{32}P as described under MATERIALS AND METHODS. The amount of ^{32}P-labeled nucleotide was below detection, i.e., less than 2 mμmoles/mg of protein per minute.

N^6-Substituted adenosine analogue	Inhibition of 0.82 μM adenosine-8-^{14}C phosphorylation		K_i	50% inhibition of growth of S-180 cells
	At 75 μM	At 150 μM		
	%	%	μM	μM
N^6-Phenyladenosine	87	97	0.6	400
N^6-*n*-Pentyladenosine	77	93		350
N^6-2-Phenoxyethyladenosine	77	91		≫300
N^6-Isoamyladenosine	62	83		280
N^6-Hexyladenosine	48	72		100
N^6-2-Ethoxyethyladenosine	24	55		>300

It is likely, therefore, that not only N^6-(Δ^2-isopentenyl)adenosine, but the other N^6-substituted adenosine analogues as well, were converted only to the monophosphate level within the cells. This has been reported to be the case for two compounds, N^6-methyladenosine and 6-methylmercapto-9-(β-D-ribofuranosyl)purine (19–21). In contrast, adenosine analogues without substitution at the 6-amino group, such as tubercidin, toyocamycin, and formycin A, are known to

be converted to triphosphates which may further be incorporated into nucleic acids (15, 16, 22).

Adenosine analogues as inhibitors of growth of S-180 cells in vitro. All the compounds in Table 1 which were substrates of the kinase, except N^6-methyl- and N^6-benzyladenosine, were also potent inhibitors of cellular multiplication. The most potent were toyocamycin and tubercidin, which are known to be incorporated into nucleic acids (15, 16). It is interesting that N^6-(Δ^2-isopentenyl)tubercidin was 200 times less cytotoxic than tubercidin itself, most probably because of the lack of incorporation into nucleic acids. It is reasonable to assume that this analogue, like N^6-(Δ^2-isopentyl)adenosine, stops at the monophosphate level and exerts its inhibition in that form. Compounds listed in Table 2 which were potent inhibitors of adenosine kinase, but were poorly or not at all phosphorylated, hardly interfered with the multiplication of S-180 cells *in vitro*.

DISCUSSION

Adenosine kinase is a "salvage" enzyme and is not needed if synthesis of AMP *de novo* is functional. Therefore, it is not surprising that the compounds listed in Table 2, which are potent inhibitors of the enzyme but are poorly or not at all phosphorylated, interfered little if at all with the multiplication of S-180 cells *in vitro*. Although this enzyme is abundant in most living systems, it has been shown to be absent from human epithelial carcinoma cells resistant to the otherwise cytotoxic agents 2-fluoroadenosine and 6-methylmercapto-9-(β-D-ribofuranosyl)-purine (23, 24). Indeed, only the sensitive cells containing adenosine kinase were found to form the phosphorylated derivatives. Recently, a mouse Sarcoma 180 cell line was developed *in vitro* which was resistant to a number of potent cytotoxic adenosine analogues and was found to contain only traces of adenosine kinase (4, 25). This prompted the present investigation of the properties and substrate specificity of the enzyme in the sensitive S-180 cells.

This study differs from some previous ones (6, 26) in utilizing a direct adenosine kinase assay instead of a coupled one. New observations provided by this study are the substrate inhibition at relatively low concentrations of adenosine (50% at 100 μM), the lack of correlation between Mg^{++} and ATP requirements, and the substrate and inhibitor specificity, especially as regards the N^6-substituted adenosine analogues. Substrate inhibition of adenosine kinase was not observed[2] by Murray (11) or by Holmsen *et al.* (28) in crude extracts of Ehrlich ascites cells or platelets, respectively. Our observations concerning the K_m for ATP (180–220 μM) and lack of significant inhibition by excess ATP compare well with studies of other workers (11, 28, 29), who also used a direct assay.[2] In contrast, Lindberg *et al.* (6) and Kornberg and Pricer (26) observed optimal adenosine phosphorylation at 500 μM ATP, while higher concentrations were inhibitory. Lindberg *et al.* (6) also observed an optimal Mg^{++}: ATP ratio of unity for the rabbit liver enzyme, in contrast to a ratio of 0.1–0.2 for the S-180 enzyme. These differences may reflect the indirect assay used by these investigators, which included an ATP-regenerating system (phosphoenolpyruvate–pyruvate kinase) and lactate dehydrogenase, and in which the oxidation of NADH to NAD^+ was measured at 340 mμ. High concentrations of ATP are known to inhibit this ATP-regenerating system (30), this inhibition being overcome by Mg^{++} (31). In addition, the substrate for pyruvate kinase is the Mg^{++}-ADP complex (32). In our studies as well as those of Lindberg *et al.* (6), the enzyme preparation contained EDTA. Indeed, no requirement for Mg^{++} has been observed in studies which employed no EDTA (7, 11). Murray (11) observed inhibition of adenosine kinase by EDTA; this could be overcome by Mg^{++}. It appears, therefore, that adenosine kinase has a requirement for Mg^{++}, which is observed only in the presence of EDTA, suggesting that Mg^{++} may normally be bound to the enzyme.

[2] After completion of this manuscript, we came across the paper by Meyskens and Williams (27). Using crude lysates of human erythrocytes and a direct assay, these authors observed no substrate inhibition at 40 μM adenosine, while concentrations of ATP higher than 1.25 mM were inhibitory.

On the basis of the present studies, the requirement for Mg^{++} is not for the formation of an Mg^{++}-ATP complex, which has been reported to be the active substrate for many other kinases (33).

The interest in N^6-substituted adenosines stems from the observation that several of these are potent cytotoxic agents (34–37), and that treatment of human myelogenous leukemia with one of them, N^6-(Δ^2-isopentenyl)adenosine, showed some degree of success (38). Also, this compound and several related analogues are potent inhibitors of adenosine uptake by mammalian cells, unrelated to the adenosine kinase content of these cells (3, 4). Furthermore, many of these nucleosides as well as the corresponding free bases are potent cytokinins in plant systems (for discussion, see ref. 39).

As regards the substrate specificity of adenosine kinase, it is interesting that the effect of replacement of the CH_3 group on the isopentenyl side chain by chlorine was entirely dependent on the position of the halogen. Thus, replacement at the *cis* position increased the rate of phosphorylation slightly while the affinity for the enzyme dropped; chlorine at the *trans* position decreased the reaction rate about 50% but doubled affinity for the enzyme.

The present study provides proof for the formation of the 5′-monophosphate of N^6-(Δ^2-isopentenyl)adenosine and the lack of its further phosphorylation by adenylate kinase. This, together with previous evidence concerning formation of the monophosphates of N^6-methyladenosine (19) and 6-methylmercapto-9-(β-D-ribofuranosyl)purine (20), suggests that all the cytotoxic N^6-substituted adenosines which are substrates of adenosine kinase stop at the monophosphate level and exert their inhibitory effect in that form. Since previously formed purines, their ribonucleosides, or deoxyribonucleosides were unable to protect the cells against the cytotoxicity of these compounds (35, 36), it must be concluded that the primary mode of action resulting in the inhibition of cellular multiplication is not feedback inhibition.

Although the present study provides useful information on the substrate specificity of adenosine kinase, the finding of potent inhibitors that possess either poor or no substrate affinity for this enzyme and at the same time lack cytotoxicity is also interesting because of the possible usefulness of such compounds against infectious organisms which lack the ability to synthesize purines *de novo* and are dependent on adenosine. These compounds may also prove useful in protection against the toxicity of compounds which require adenosine kinase for activation.

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