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N^6-(Δ^2-Isopentenyl)adenosine 5′-Monophosphate: Formation and Effect on Purine Metabolism in Cellular and Enzymatic Systems

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SUMMARY

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The growth of Sarcoma 180 cells (S-180), whether dependent on the synthesis of purine nucleotides *de novo* (folate medium) or on preformed purines (amethopterin medium) such as hypoxanthine, adenine, or adenosine, was inhibited by 50 and 100% at 15–22 and 100 μM N^6-(Δ^2-isopentenyl)adenosine, respectively. This adenosine analogue was shown to accumulate in the cell water in the form of the 5′-monophosphate at 2 mM when the extracellular nucleoside level was 100 μM. In S-180 cells incubated with 100 and 300 μM N^6-(Δ^2-isopentenyl)adenosine and 30 μM [8-^{14}C]adenine the formation of [8-^{14}C]ATP was inhibited by 55 and 64%, respectively, and [8-^{14}C]AMP accumulated at 53 μM as compared with the control, where AMP was undetectable. Under similar conditions the conversion of 100 μM [8-^{14}C]hypoxanthine to ATP and GTP was inhibited by 51 and 65%, respectively. A study was made on the effect of chemically prepared N^6-(Δ^2-isopentenyl)adenosine 5′-monophosphate on several enzymes involved in the metabolism of purine nucleotides, using cell-free extracts of S-180 cells. This compound inhibited adenosine kinase by competing with ATP (K_i/K_m, 1.4), adenine phosphoribosyltransferase by competing with 5-phosphoribosyl 1-pyrophosphate (K_i/K_m, 4.0), AMP kinase by competing with AMP (K_i/K_m, 8.8), IMP dehydrogenase by competing with IMP (K_i/K_m, 15), and adenylosuccinate synthetase noncompetitively with respect to IMP (K_i, 3.0 mM). This nucleotide analogue was also a moderate inhibitor of 5-phosphoribosyl 1-pyrophosphate synthetase, and a poor inhibitor of adenylosuccinate lyase, hypoxanthine phosphoribosyltransferase, and guanylate kinase. Since the multiplication of S-180 cells *in vitro* is independent of preformed purines, it appears that the inhibition of adenine phosphoribosyltransferase or of adenosine kinase cannot be the basis for the cytotoxicity of N^6-(Δ^2-isopentenyl)adenosine. The intracellular pools of AMP and IMP were normally quite low (much less than 0.1 mM) as compared with that of N^6-(Δ^2-isopentenyl)adenosine 5′-monophosphate. It appears, therefore, that AMP kinase and IMP dehydrogenase may be the critical sites of action. Inhibition of these enzymatic steps could cause a shortage of ATP and GTP, leading to inhibition of all the synthetic processes requiring these triphosphates and, eventually, to cell death.

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INTRODUCTION

N^6-(Δ^2-Isopentenyl)adenosine is the 9-β-ribosyl derivative of one of the minor bases found in tRNA of all species. While this nucleoside analogue acts as a cytokinin in plant systems, it is cytotoxic to most mammalian cells (1, 2) and has shown some success in the chemotherapy of a patient with promyelocytic leukemia (3).

In cell-free systems N^6-(Δ^2-isopentenyl)-adenosine has been shown to be a substrate for adenosine kinase (K_m, 11 μM) (4). It was a poor inhibitor of adenosine deaminase (K_i, 1.3 mM) (5), glucose 6-phosphate dehydrogenase (K_i, 3–5 mM) (6) and mammalian tRNA methylase (7).

In Sarcoma 180 cells N^6-(Δ^2-isopentenyl)-adenosine was found to be a potent inhibitor for the uptake of purine and pyrimidine nucleosides (8, 9). The normally observed increase in uridine kinase activity caused by phytohemagglutinin was prevented in human lymphocytes when exposed to this adenosine analogue (10). The cytotoxicity of N^6-(Δ^2-isopentenyl)adenosine for S-180[2] cells was shown to correlate with the intracellular capacity of these cells to convert the analogue to the 5′-monophosphate; in cells resistant to the analogue both its uptake and phosphorylation were drastically reduced (5). On the basis of these observations it appeared that the 5′-monophosphate of N^6-(Δ^2-isopentenyl)adenosine was the actual cytotoxic agent. The present study was undertaken to determine the effect of this 5′-monophosphate on purine nucleotide metabolism both within intact S-180 cells and in cell-free extracts. A unique method for the determination of purine nucleotide pools, especially suitable for cell culture, is also described. Abstracts on this subject have been published (11, 12).

MATERIALS AND METHODS

Compounds. [8-^{14}C]N^6-(Δ^2-Isopentenyl)-adenosine was prepared by Dr. M. Fleysher in this department (13) and was 99.7 % pure. The other labeled compounds were obtained from Amersham/Searle and Schwarz/Mann. [8-^{14}C]IMP, obtained from Schwarz/Mann, contained inosine, which was removed by column chromatography on DEAE-cellulose (formate). [U-^{14}C]L-Aspartate (Amersham/Searle) contained a 0.5 % impurity of β-alanine, which did not interfere with these studies and therefore was not removed. The purity of the other labeled compounds was adequate without purification. Rabbit muscle adenylate kinase (grade III) and 5′-nucleotidase of *Crotalus adamanteus* venom (grade II) were obtained from Sigma Chemical Company.

Chemical synthesis of IPAMP. Phosphorylation of IPAdo to the 5′-monophosphate was carried out as described by Yoshikawa *et al.* (14). One millimole of IPAdo was dissolved in 2.5 ml of trimethyl phosphate. The solution was cooled to 0° in ice, and 0.36 ml of $POCl_3$ was added. After mixing, the solution was allowed to stand in ice for 6 hr, diluted with 50 ml of ice-cold water, brought to about pH 2 with 0.1 N NaOH, and then extracted once with 40 ml of cold ethyl ether, which was discarded. The solution was brought to room temperature, and charcoal (Norit A) was added. After 10 min the suspension was filtered through Whatman No. 5 filter paper on a Buchner funnel, and the filtrate was discarded. The charcoal on the paper was washed five or six times with water, and the adsorbed compounds were then eluted with a mixture of water–NH_4OH–ethanol (50:2:48). The solution was evaporated to dryness under vacuum at 30°, and the residue was dissolved in 5 ml of water and placed on a 1.5 × 15 cm column of DEAE-cellulose (formate). The column was washed with 200 ml of 1 mM formic acid to remove the unreacted IPAdo, and IPAMP was eluted with about 150 ml of 0.1 M formic acid. Formic acid was removed by evaporation under vacuum at 30°, and the residue was dissolved in 100 ml of water and evaporated again to dryness. The residue was dissolved in water and stored frozen at −70°. The molarity of the solution was calculated from the extinction at 267 nm (15). The yield of the nucleotide

[2] The abbreviations used are S-180, Sarcoma 180 cells; IPAMP, N^6-(Δ^2-isopentenyl)adenosine 5′-monophosphate; IPAdo, N^6-(Δ^2-isopentenyl)-adenosine; PRPP, 5-phosphoribosyl 1-pyrophosphate; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid.

was more than 40%. Inorganic and organic phosphates were determined by the method of Dryer *et al.* (16).

Cell extracts. The cell line was the parent mouse Sarcoma 180 grown in roller bottles, harvested, and stored at −70°, as described (5). They were generally used within a month, and the cell extracts were prepared at 0–4° by either of two procedures.

Procedure A: The frozen cells were thawed and suspended in 1 volume of buffer, different buffers being used for the different enzyme preparations (Table 1). The cells were then homogenized in a Potter-Elvehjem homogenizer, diluted with 2 volumes of the same buffer, and centrifuged at 105,000 × *g* for 60 min in a Beckman model L2-65B ultracentrifuge. The supernatant fluid was dialyzed against 200 volumes of the same buffer for 16 hr and recentrifuged for 30 min to remove the precipitate that formed. The supernatant fraction was stored at −70° in small aliquots, which were used within a month. Protein in the extract was assayed by the method of Lowry *et al.* (18), using bovine serum albumin as the standard.

Procedure B: The frozen cells were thawed and suspended in 3 volumes of buffer at room temperature in a stoppered polypropylene tube. The tube was inverted gently five or six times and placed in the −70° storage chest for 60 min. The suspension was then thawed in ambient water and mixed as above. The freezing-thawing procedure was repeated two more times; the suspension was then centrifuged, and the extract was dialyzed, recentrifuged, assayed for protein, and stored as in procedure A.

Enzyme assays. The enzyme assays using the crude or partially purified cell extracts were based on conversion of the ^{14}C-labeled substrates to products which were estimated after chromatographic separation (Table 1). In each case the assay mixture and enzyme were first incubated separately for 3 min at 35°, after which the reaction at 35° was started by addition of the enzyme. The reaction was stopped either by immersing the assay tubes into boiling water for 2 min, by the addition of 0.1 volume of cold 30% trichloracetic acid, or by diluting the reaction mixture with 50 ml of ice-cold water (Table 1). The mixtures were cooled in ice and centrifuged to remove the precipitated protein. An aliquot (50 μl) of the supernatant fraction was spotted on a paper strip and chromatographed as described below. The reaction rates represented initial velocities; the conversion of substrate in the uninhibited control was always less than 20%.

Radiochromatographic techniques. Separation of radiolabeled substrates from products was carried out either on Whatman No. 3MM or DE81 paper strips (2.5 × 60 cm) by descending chromatography at room temperature (Table 1). The following solvents were used (the figures in parentheses indicate the time required by the solvent to move about 50 cm): solvent 1, isobutyric acid–NH_4OH–water, 66:1:33 (16 hr); solvent 2, butanol–acetic acid–water, 50:25:25 (18 hr); solvent 3, 2-propanol–NH_4OH–water, 70:10:20 (22 hr); solvent 4, saturated $(NH_4)_2SO_4$ solution–water–2-propanol 79:19:2 (12 hr); solvent 5, butanol–acetic acid–water, 20:3:7 (4 hr, 18-cm run). Unlabeled authentic compounds (20 μl of a 10 mM solution) were used as internal markers for detection under ultraviolet light. The paper strips were cut into 1-cm sections and counted as described (5) by first scanning for ^{14}C content for 1 min each and then recounting the labeled sections to obtain a minimum of 10,000 counts/section.

Inhibition of purine biosynthesis de novo. The method described by Henderson (19) was adopted with minor modifications. Monolayers of S-180 cells in T-15 flasks, prepared as described (5), were first incubated with 12 μM azaserine and varied concentrations of the nucleoside analogue for 30 min at 36°. After the addition of 40 μl of 100 mM glutamine and 50 μl of 80 mM [2-^{14}C]glycine (0.5 μCi/μmole), the incubations were continued for 60 min. The medium was poured out, the flask was set on ice, and the cell layer was rinsed twice with 2 ml of ice-cold, glutamine-free medium. Aliquots (50 μl) of the medium and wash were counted for ^{14}C. The cell layer was then extracted with 2 ml of ice-cold 5% trichloracetic acid, the remaining cells were dissolved in 0.2 N NaOH, and both were counted for ^{14}C as described

TABLE 1

Enzyme preparation and assay conditions

Enzyme	Cell extract prepared using		8-^{14}C-Labeled substrate	R_F[b]	Radioactive products	R_F[b]	Reaction stopped by	Paper chromatography	
	Buffer[a]	Procedure						Whatman No.	Solvent[c]
Adenosine kinase	1	A[d]	Adenosine	0.4	AMP + ADP	0.03	Heat	3MM	5
Adenine phosphoribosyl transferase	1	A	Adenine	0.9	AMP	0.5	Heat	3MM	1
Hypoxanthine phosphoribosyltransferase	1	A	Hypoxanthine	0.6	IMP	0.2	Heat	3MM	1
Adenylate kinase	1	A[d]	AMP	0.5	ADP	0.3	CCl_3COOH	3MM	1
Guanylate kinase	1	A	GMP	0.2	GDP, GTP	<0.04	CCl_3COOH	3MM	2
Adenylosuccinate synthetase	10 mM HEPES-NaOH, pH 7.4	B[e]	IMP	0.1	Succinyl-AMP	<0.03	CCl_3COOH	DE81	1
Adenylosuccinate lyase	1	B	AMP	0.3	Succinyl-AMP	<0.03	CCl_3COOH	DE81	1
Iosinate dehydrogenase	1	B	IMP	0.6	XMP	0.4	CCl_3COOH	DE81	4
5-Phosphoribosyl-pyrophosphate synthetase	50 mM potassium phosphate (pH 7.4) + 25 mM glutathione	B	[^{14}C] Ribose-5-P		PRPP		Ice-cold water	—[f]	—[f]

[a] Buffer of the same composition was used for dialysis except in the case of PRPP synthetase, when glutathione was omitted. In that case 10 mM glutathione was added to the dialyzed extract before storage at −70°. Buffer 1 was 5 mM Tris-chloride, pH 7.0, containing 1 mM EDTA.

[b] R_F values are approximate, since the chromatography was carried out at room temperature (18–26°). For separation of succinyl-AMP from IMP the temperature had to be maintained above 24°.

[c] For description of solvents, see MATERIALS AND METHODS.

[d] Partial purification of adenosine kinase was carried out as described earlier (5). This preparation also contained adenylate kinase and was used for that purpose.

[e] These cells had been stored at −70° for more than 1 year. The enzyme was partially purified by streptomycin precipitation. One volume of streptomycin sulfate (5% solution in 10 mM HEPES-NaOH, pH 7.4) was added to 3 volumes of cell extract. After 10 min the solution was centrifuged at 10,000 rpm for 10 min, and the supernatant solution was used for the enzyme assay.

[f] After the reaction had been stopped by dropping the open assay tube in 50 ml of ice-cold water in a mixing cylinder (mixing 10 times by inversion), the solution was passed three times through two DE81 paper discs (4 cm in diameter) in a Millipore apparatus as described (17). The discs were washed once with 100 ml of 50 mM NH_4HCO_3 and 100 ml of water and dried, and the radioactivity was counted as described under MATERIALS AND

(5). One milliliter of the trichloracetic acid extract was placed on a 1 × 5 cm column of Dowex 1-IX-10 (formate, 400 mesh). The column was washed with 30 ml of 0.5 M formic acid, and the formylglycinamide ribonucleotide was then eluted with 25 ml of 4 M formic acid. The eluate was evaporated to dryness under vacuum, redissolved in 1 ml of water, and counted for ^{14}C.

Testing for growth inhibition. Monolayer cultures of S-180 cells were used for these studies as described previously (4).

Determination of adenine nucleotide pools. S-180 cells were grown for 9–13 days in Eagle's medium (20) supplemented with 1 μM amethopterin, 30 μM thymidine, 100 μM glycine, and 100 μM [8-^{14}C]adenine or [8-^{14}C]-hypoxanthine (1.0 μCi/μmole). Advantage was taken of the fact that under these conditions the synthesis of purine nucleotides *de novo* is completely blocked and the cells are totally dependent on an extracellular source of purines (21, 22). Adenine nucleotides, totally derived from the extracellular purines and soluble in ice-cold 5% trichloracetic acid, were analyzed and estimated as nanomoles per milligram of protein by paper chromatography, using solvent 1, as described (5). Solvent 1 failed to separate ATP from GTP and GDP. When rechromatographed using solvent 4, this spot was found to contain 6–7% of GTP and GDP; no attempt was made to resolve the latter two any further. In these cells 1 mg of protein corresponds to 7.6 mg of cell water (23); this permitted the results to be expressed as millimolar concentration in cell water.

Cellular metabolism of N^6-(Δ^2-isopentenyl)-adenosine. This study was performed using monolayers of S-180 cells and [8-^{14}C]IPAdo as described previously (5).

Effect of N^6-(Δ^2-isopentenyl)adenosine on cellular metabolism of preformed purines. Monolayers of S-180 cells in T-15 flasks (5) were first incubated for 60 min in the presence or absence of IPAdo. [8-^{14}C]Adenine (30 μM, 30 μCi/μmole) or [8-^{14}C]hypoxanthine (100 μM, 10 μCi/μmole) was then added, and the incubations were continued for 0.5–4 hr. At the end of the incubations the trichloracetic acid-soluble pools were analyzed as above.

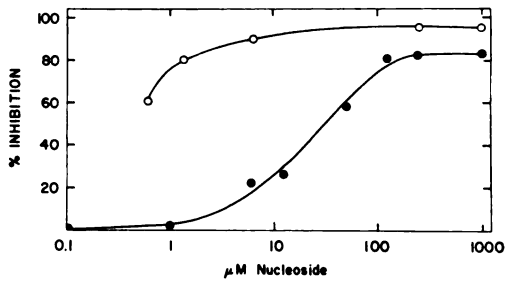

FIG. 1. *Effect of N^6-(Δ^2-isopentenyl)adenosine* (●) *and 6-methylthioinosine* (○) *on formation of formylglycinamide ribonucleotide from [2-^{14}C]glycine in S-180 cells*

For experimental details, see MATERIALS AND METHODS. Formylglycinamide ribonucleotide accumulation in the inhibitor-free control, carried in duplicate, was 2800 ± 100 cpm/0.8 mg of protein.

This investigation was supported in part by Research Grant CAO-4175 from the National Cancer Institute.

RESULTS

Inhibition of purine biosynthesis de novo. The formation of labeled formylglycinamide ribonucleotide from [2-^{14}C]glycine (in 60 min) was markedly inhibited in S-180 cells which had been incubated for 30 min with IPAdo in serum-free Eagle's medium (20). Maximal inhibition (about 80%) was observed when the IPAdo concentration was 100 μM or over (Fig. 1). The inhibitory potency of 6-methylthioinosine in S-180 cells was comparable to that observed previously in Hep-2 cells (24), and this compound was about 100 times more potent than IPAdo (Fig. 1). Neither the cellular uptake of glycine nor its incorporation into acid-insoluble material was affected by IPAdo.

Lack of effect of preformed purines on growth inhibition by IPAdo. S-180 cells were tested for growth inhibition by IPAdo in two types of media (Fig. 2). In folic acid medium the cellular purines and their nucleotides are synthesized *de novo*. In amethopterin medium (medium supplemented with 1 μM amethopterin, 30 μM thymidine, 100 μM glycine, and 100 μM preformed purine) the growth of cells is totally dependent on the preformed purine in the medium (21, 22). Figure 2 shows how the sensitivity of the cells to IPAdo during 6 days of growth was

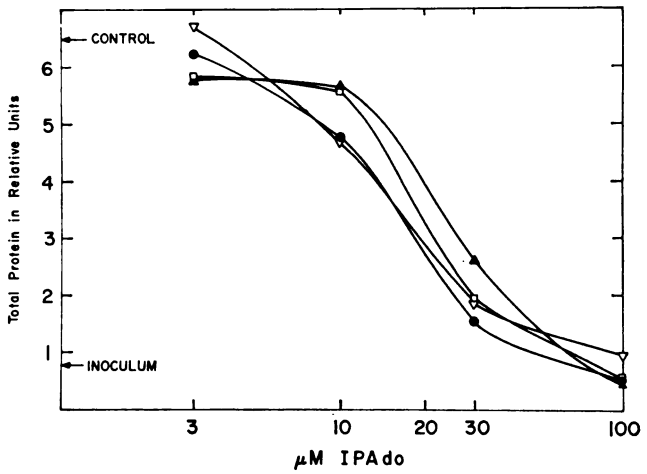

FIG. 2. *Lack of protection of S-180 cells by preformed purines against growth inhibition exerted by N^6-(Δ^2-isopentenyl)adenosine*

The cells were grown as monolayers in folic acid medium (20)(●) and in a medium containing 1 μM amethopterin to block purine biosynthesis *de novo* (21, 22), supplemented with 30 μM thymidine, 100 μM glycine, and 100 μM purine (▽, adenine; □, hypoxanthine; ▲, adenosine). Growth was continued for 6 days, the medium was changed twice, and the amount of cells was estimated by protein assay (18).

unaffected by the availability of preformed purines, suggesting that inhibition of the biosynthesis of purine nucleotides *de novo*, observed above, may not be responsible for inhibition of growth.

As in amethopterin medium, no protection of S-180 cells against IPAdo was afforded if Eagle's medium (no amethopterin) was supplemented with hypoxanthine or adenine. However, increasing the concentration of adenosine afforded some protection. Thus, in the presence of 300 μM adenosine, a 10-fold higher concentration of IPAdo was required for inhibition. This observation is consistent with our earlier finding that IPAdo is a competitive inhibitor of adenosine uptake (K_i, 1.4 μM) (8) as well as of adenosine phosphorylation (K_i, 15 μM) (4).

Effect of IPAdo on adenine nucleotide pools of S-180 cells. Table 2 lists the percentage composition of the adenine nucleotide pools of S-180 cells and also indicates the millimolar concentration in cell water of each nucleotide derived from [^{14}C]adenine. These values are in good agreement with values obtained by different methods and reported for mouse tumor cells (25–27).

The method used here for analyzing the pools has not been previously reported. Although ATP was the major component (89%), AMP constituted less than 1% of the total; in half the cases analyzed, no AMP was detected. It is of interest that [8-^{14}C]-adenine was found at 130 μM in cell water when the extracellular concentration of the

TABLE 2

Adenine nucleotide pools in S-180 cells

S-180 cells were maintained for 9–13 days in amethopterin medium (see MATERIALS AND METHODS) supplemented with [8-^{14}C] adenine; the cold trichloracetic acid-soluble pool was analyzed by paper chromatography using solvent 1. The data were derived from nine independent experiments, except for adenine and AMP, which are based on eight experiments. The cells were quantitated by protein assay. In these cells 1 mg of protein corresponds to 7.6 mg of cell water (23).

[8-^{14}C]-Adenine metabolite	Percentage of total	Concentration in cell water	
		Mean ± SE	Range
		mM	*mM*
ATP[a]	89.3	4.3 ± 0.22	3.3–5.0
ADP	7.1	0.34 ± 0.037	0.19–0.58
AMP	0.90	0.043 ± 0.019	0–0.13
Adenine	2.7	0.13 ± 0.025	0–0.19

[a] As described in the text, the ATP spot, when rechromatographed using solvent 4, was found to contain 6% GTP + GDP.

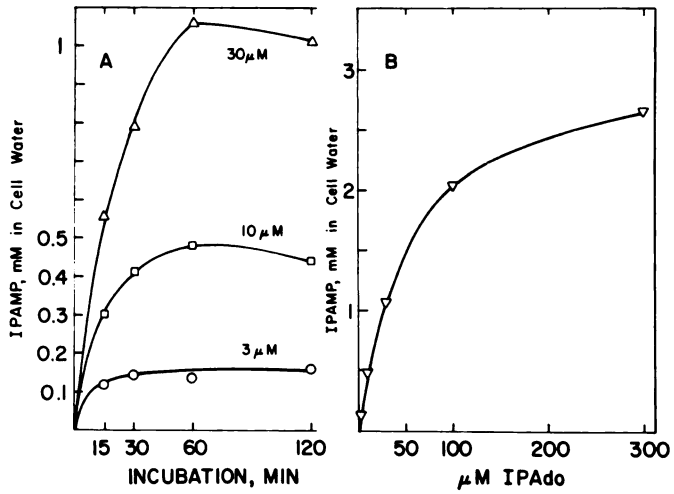

FIG. 3. *Cellular conversion of N^6-(Δ^2-isopentenyl)adenosine to IPAMP*

A. S-180 cells were incubated with [8-^{14}C]IPAdo (180,000 cpm/ml) for 15 min–2 hr and analyzed as described under MATERIALS AND METHODS. ○, 3 μM IPAdo; □, 10 μM; △, 30 μM. B. Concentration of 5′-IPAMP in S-180 cell water after incubation for 60 min at the indicated concentrations of [8-^{14}C] IPAdo.

precursor adenine was 100 μM. This finding supports the view that purine bases are taken up by mammalian cells by facilitated diffusion (28).

When S-180 cells, grown for at least 7 days in amethopterin medium (see MATERIALS AND METHODS) containing 100 μM [8-^{14}C]-hypoxanthine, were incubated for 1 hr in the same medium supplemented with 100 μM IPAdo, the ATP pool was reduced by 30%. Under similar conditions, but using [8-^{14}C]-adenine in place of hypoxanthine, incubation for 5 hr with 100 μM IPAdo resulted in a 46% reduction in ATP pool.

N^6-(Δ^2-Isopentenyl)AMP accumulation in S-180 cells. The identity of 5′-IPAMP formed in S-180 cells from N^6-(Δ^2-isopentenyl)adenosine was established previously (5). The intracellular level of IPAMP reached a plateau after about 60 min when incubated with 3–30 μM [8-^{14}C]-IPAdo (Fig. 3A). The millimolar concentration of IPAMP in cell water at 60 min was related to the extracellular IPAdo concentration (Fig. 3B). At about 300 μM IPAdo in the medium the cellular IPAMP approached a plateau level of 3 mM in cell water. At 60 min, depending on the extracellular concentration of [8-^{14}C]IPAdo, 61–79% of the total trichloracetic acid-soluble radioactivity was in the form of IPAMP. In contrast, the unaltered nucleoside was found at low levels, 150 and 330 μM in cell water, when the extracellular levels were 100 and 300 μM, respectively. When these results are compared with the data in Fig. 2, one can estimate that at 50% growth-inhibitory concentrations of IPAdo in the medium, the intracellular IPAMP was 600–900 μM, and under 100% inhibitory conditions it was about 2 mM.

Effect of IPAdo on cellular metabolism of [8-^{14}C]adenine and hypoxanthine. Since it was established that a plateau level of intracellular IPAMP was reached in 60 min (see above), this time period was chosen for the length of preliminary incubation of cells with IPAdo. The labeled purine precursor was added only thereafter. Both 100 and 300 μM IPAdo (corresponding to 2 and 2.6 mM intracellular IPAMP) inhibited the formation of ATP from 30 μM [8-^{14}C]adenine by 55 and 64%, respectively (Fig. 4A). At the same time [8-^{14}C]AMP, which was undetectable in the controls, was found at 53 μM in cell water after 4 hr of incubation with 300 μM IPAdo (Fig. 4B). In a similar study the conversion of 100 μM hypoxanthine to ATP was inhibited by 51 and 65% at 100 and 300 μM IPAdo, respectively.

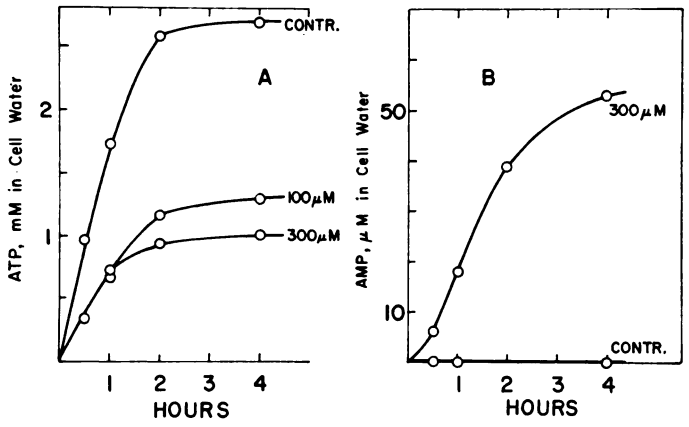

FIG. 4. *Effect of N^6-(Δ^2-isopentenyl)adenosine on cellular metabolism of adenine*

Monolayers of S-180 cells were incubated for 60 min at 36° with 100 and 300 μM IPAdo (see MATERIALS AND METHODS). This medium was then supplemented with 30 μM [8-^{14}C]adenine (30 μCi/μmole), and the incubation was continued for 0.5–4 hr. Control, no IPAdo: the material soluble in cold trichloracetic acid was analyzed by paper chromatography using solvent 1. A. Data for ATP. As explained in the text, the ATP spot contained 7% of GTP + GDP as revealed by rechromatography with solvent 4. B. Data for AMP.

Characterization of synthetic 5′-IPAMP. The synthetic IPAMP moved as a single band on Whatman No. 3MM paper in solvent systems 1, 3, and 5, and the movement differed from that of IPAdo. R_F values for IPAMP and IPAdo were, respectively, 0.8 and 0.93 in solvent 1, 0.5 and 0.9 in solvent 3, and 0.3 and 0.8 in solvent 5. The ultraviolet absorption spectra at pH 7.0 and the A_{290}:A_{260} ratios (0.29) for the nucleotide and IPAdo were identical. The product obtained on treatment of IPAMP with 5′-nucleotidase was chromatographically indistinguishable from IPAdo. No inorganic phosphate was detected when the preparation (0.6 μmole) was analyzed by the method of Dryer *et al.* (16). On treatment with 88 μg of snake venom 5′-nucleotidase in 62.5 mM Tris-chloride, pH 8.9 (30 min, 35°), 0.4 μmole of IPAMP gave 0.38 ± 0.02 μmole of phosphate (average of three determinations ± standard deviation). On digestion with boiling 5 N H_2SO_4 for 4 hr, 0.2 μmole of IPAMP gave 0.22 ± 0.03 μmole of phosphate (average of three determinations ± standard deviation). Thus the phosphate group in the compound was located in the 5′-position and IPAdo:P_i ratio was approximately 1:1, proving that the synthetic material was indeed the 5′-monophosphate of IPAdo.

Comparison of cell extraction procedures. Cell extracts prepared by homogenization (procedure A) were suitable for studies on adenine phosphoribosyltransferase, hypoxanthine phosphoribosyltransferase, and guanylate kinase. However, the study of the other enzymes was hampered by the presence of phosphatases and/or nucleotidases in these extracts. Release of these catabolic enzymes was avoided by breaking the cells by slow freezing and thawing (procedure B). Thus extract B (1 mg of protein per 0.4 ml) hydrolyzed less than 1% of 125 μM AMP or IMP when incubated for 60 min at 35° in 50 mM potassium phosphate, pH 7.4; this was in contrast to extract A, which under similar conditions hydrolyzed 30–40%.

Adenine phosphoribosyltransferase (AMP: pyrophosphate phosphoribosyltransferase, EC 2.4.2.7). The enzyme assay was similar to that described by Murray (29). The conversion of 125 μM adenine to AMP by crude extracts of S-180 cells (11–53 μg of protein per 0.4 ml) was linear for at least 10 min and depended on PRPP. Over 99% of total radioactivity in the chromatograms was associated with adenine and AMP peaks. In the presence of 125 μM adenine and 12.5 μM PRPP, 125 μM IPAMP inhibited the reaction by about 50% but had no effect in the presence of 12.5 μM adenine and 250 μM PRPP.

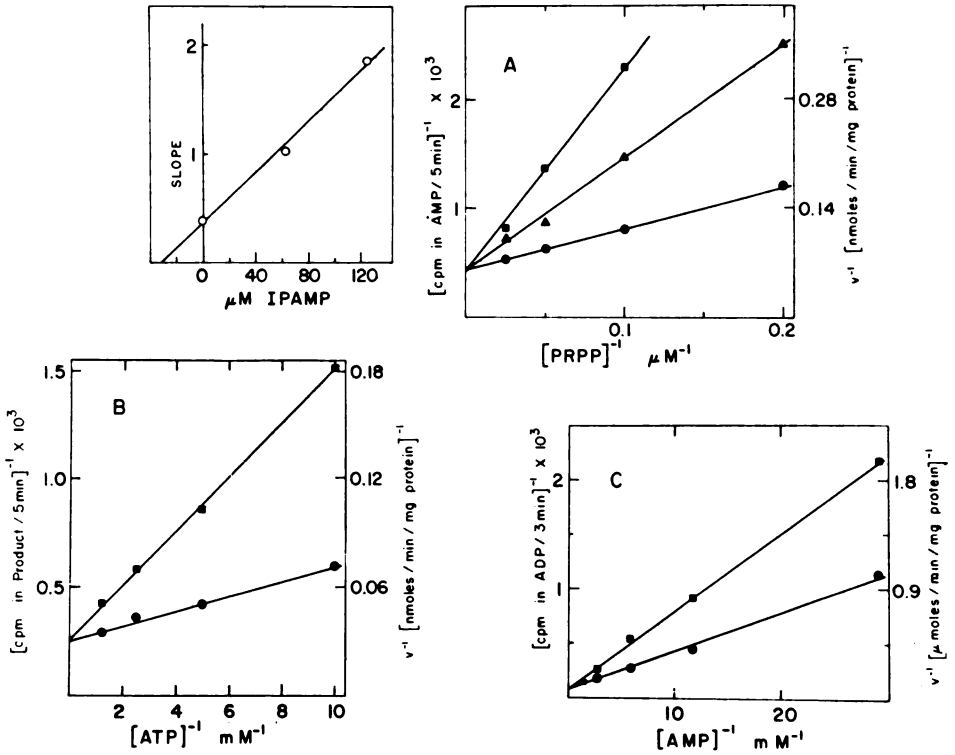

FIG. 5. *Double-reciprocal plots (30) for inhibition of adenine phosphoribosyltransferase, adenosine kinase, and AMP kinase by IPAMP*

A. Adenine phosphoribosyltransferase. The slope is expressed in relative units. The reaction mixture (0.4 ml) contained 125 μM [8-^{14}C]adenine (8 μCi/μmole), 2.5 mM $MgCl_2$, 5–40 μM PRPP, 50 mM Tris-chloride (pH 7.8), and cell extract equal to 21 μg of protein, and was incubated for 5 min at 35°. For details, see the text. ●, no IPAMP; ▲, 62.5 μM; ■, 125 μM. K_m for PRPP, 8 μM; K_i for IPAMP, 32 μM; V_{max}, 16 nmoles/min/mg of protein.

B. Ade nosine kinase. The reaction mixture (0.4 ml) contained 4 μM [8-^{14}C]adenosine (50 μCi/μmole), 0.25 mM $MgCl_2$, 100–800 μM ATP, 50 mM potassium phosphate (pH 7.0), and enzyme partially purified from S-180 cells (4) equal to 2.6 μg of protein. IPAMP-free samples were incubated for 5 min, and IPAMP-containing samples, for 10 min. For details, see the text. ●, no IPAMP; ■, 0.5 mM. K_m for ATP, 140 μM; K_i for IPAMP, 200 μM; V_{max}, 33 nmoles/min/mg of protein.

C. AMP kinase of S-180 cells. The reaction mixture (0.5 ml) contained 40–400 μM [8-^{14}C]AMP (2 μCi/μmole), 2.5 mM $MgCl_2$, 2.5 mM ATP, 50 mM potassium phosphate (pH 7.0), and the same enzyme preparation as in B, equal to 2.6 μg (20 μl) of protein (enzyme was diluted 1:5 in a solution of 20 mM cysteine, 50 mM potassium phosphate, pH 7.0, and 1 mg/ml of albumin). The mixture was incubated for 3 min. For details, see the text. ●, no IPAMP; ■, 3.6 mM. K_m for AMP, 0.4 mM; K_i for IPAMP, 3.5 mM; V_{max}, 14 μmoles/min/mg of protein.

Thus IPAMP was competitive with PRPP (Fig. 5A). The K_m for PRPP was 8 μM, in good agreement with the value of 7–12 μM reported for the enzyme in Ehrlich ascites cells (29). The K_i value for IPAMP was 32 μM, about 4 times the K_i for AMP (29). Even a 3.8 mM concentration of the nucleoside (IPAdo) failed to interfere with the formation of AMP from 125 μM adenine and PRPP.

Hypoxanthine phosphoribosyltransferase (IMP:pyrophosphate phosphoribosyltransferase, EC 2.4.2.8). The activity of hypoxanthine phosphoribosyltransferase in crude extracts of S-180 cells was similar to that of adenine phosphosphoribosyltransferase when tested under identical conditions (15 nmoles/mg of protein per minute). At 11–53 μg of protein per 0.4 ml, the conversion of 125 μM hypoxanthine to IMP was also linear for 10

min and depended on PRPP. IPAMP at 625 μM, had no effect, even when the concentration of PRPP or hypoxanthine was 12.5 μM, and at 2.5 and 5 mM it inhibited the reaction only by 10 and 30%, respectively, in the presence of 125 μM PRPP and hypoxanthine. It is of interest that the two functionally related enzymes, adenine- and hypoxanthine-PRPP transferases, differed from each other in that IPAMP competed with PRPP for the adenine- but not for the hypoxanthine-metabolizing enzyme.

Adenosine kinase (ATP:adenosine 5'-phosphotransferase, EC 2.7.1.20). IPAdo itself was shown to be a substrate of partially purified adenosine kinase (K_m, 11 μM) and thus competitively inhibited the phosphorylation of adenosine (K_m, 0.5 μM) with a K_i of 15 μM (4). Using similarly purified enzyme, the product of the reaction, 5'-IPAMP, was also found to inhibit adenosine phosphorylation (Fig. 5B), but in this case by competing with ATP. The K_i for IPAMP (200 μM) was somewhat higher than the K_m for ATP (140 μM). Unlike IPAdo, the nucleotide analogue did not compete with adenosine, since no inhibition was observed at 1 mM IPAMP, 4 μM adenosine, and 5 mM ATP.

AMP kinase (ATP:AMP phosphotransferase, EC 2.7.4.3). AMP kinase was assayed by a modified procedure of Noda (31), using the same enzyme preparation as for adenosine kinase. Under these conditions the conversion of 120 μM [8-^{14}C]AMP to ADP was linear for at least 3 min and no formation of radioactive ATP was detected. The reaction was dependent on ATP. IPAMP competed with AMP (Fig. 5C); the K_i and K_m values were 3.5 and 0.4 mM, respectively. For crystalline rabbit muscle AMP kinase the K_m and K_i values for AMP and IPAMP were 0.7 and 6.1 mM, respectively. Both K_m values for AMP are comparable with the values reported for the rabbit muscle enzyme (31, 32). While 4.2 mM IPAMP in the presence of 400 μM AMP inhibited S-180 AMP-kinase by 30%, 4 mM IPAdo had no effect.

IMP dehydrogenase (IMP:NAD$^+$ oxidoreductase, EC 1.2.1.14). The assay conditions of Anderson and Sartorelli (33) were modified for the present purpose. The oxidation of [8-^{14}C]IMP to XMP was measured after chromatographic separation in solvent 4 (see MATERIALS AND METHODS). Since solvent 4 does not separate XMP from inosine, the identity of the product was examined by further chromatography of the XMP spot in solvent 1. No inosine was detected. The formation of XMP from IMP depended on NAD$^+$, and the reaction was linear for at least 45 min at 1–4 mg of protein per milliliter. At 0.1 mM IMP and 1.25 mM NAD$^+$, 1.3 and 6.5 mM IPAMP inhibited XMP formation by 40 and 78%, respectively. At 1.25 mM IMP and 0.1 mM NAD$^+$, the same concentrations of IPAMP again inhibited the reaction by 25 and 78%, respectively. Thus the nucleotide analogue seemed to compete not only with IMP but with NAD$^+$ as well. The competition was investigated only with respect to IMP (Fig. 6A). The K_i value for IPAMP was 200 μM, and the K_m for IMP was 13 μM, similar to the constants for the enzyme in Ehrlich ascites (17) and S-180 cells (33). The concentration of NAD$^+$ (1.25 mM) used in the latter experiments is known to inhibit the reaction by about 30% (33) but was chosen to eliminate the effect of possible competition between IPAMP and NAD$^+$. IPAdo at 4.5 mM inhibited the oxidation of 0.1 mM IMP only about 10% at 1.25 mM NAD$^+$.

Adenylosuccinate synthetase [IMP:L-aspartate ligase (GDP), EC 6.3.4.4]. Extracts of freshly harvested S-180 cells could not be used because of the presence of adenylosuccinase, while extracts of cells stored at −70° for more than 1 year were essentially free of this interference. Similar selective adenylosuccinase inactivation with the age of the preparation has been reported earlier (34). The synthetase was assayed by a modified procedure of Lieberman (35). The enzyme activity was greatly affected by the type of buffer (50 mM, pH 7.4) and diminished in the order HEPES-NaOH > glycine-NaOH > Tris-chloride > potassium phosphate. HEPES-NaOH was chosen for both the cell extraction and the enzyme assay. The cell extract was partially purified by streptomycin precipitation (Table 1), and streptomycin sulfate (150 μM) was also added to the

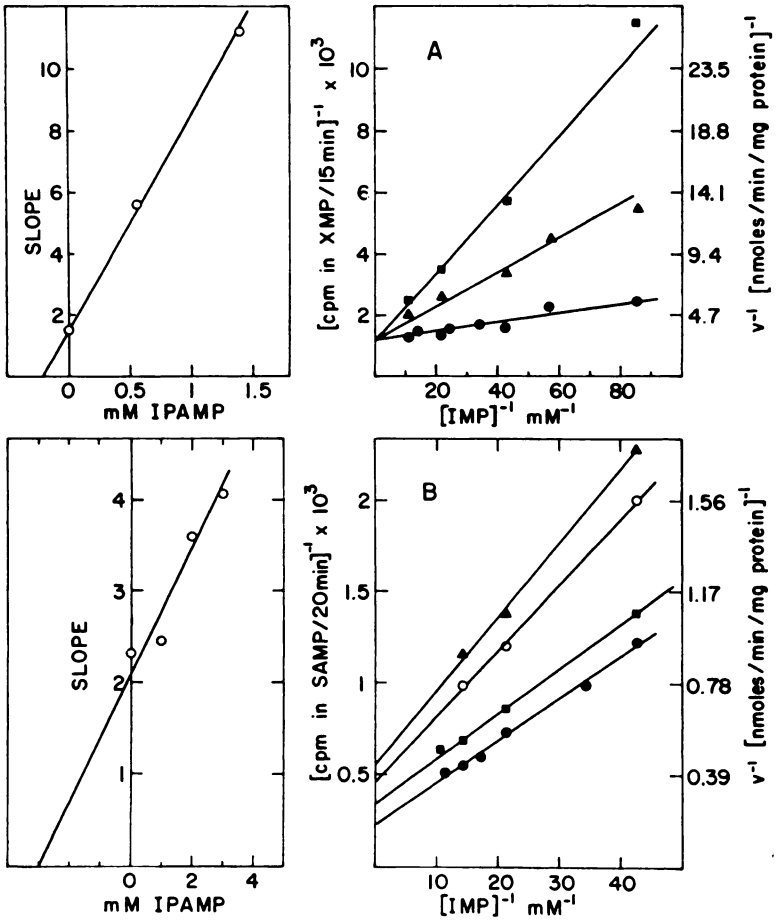

FIG. 6. *Double-reciprocal plots for inhibition of IMP dehydrogenase and adenylosuccinate synthetase by IPAMP*

A. IMP dehydrogenase. Slopes are expressed in relative units. The reaction mixture (0.4 ml) contained 12–100 μM [8-^{14}C]IMP (2.5 μCi/μmole), 1.25 mM NAD^+, 63 mM potassium phosphate (pH 7.4), and cell extract equal to 0.4 mg of protein, and was incubated for 15 min. For details, see the text. ●, no IPAMP; ▲, 0.55 mM; ■, 1.4 mM; K_m for IMP, 13 μM; K_i for IPAMP, 0.22 mM; V_{max}, 0.36 nmole/min/mg of protein.

B. Adenylosuccinate synthetase. The reaction mixture (0.4 ml) contained 23–400 μM [8-^{14}C]IMP (2.5 μCi/μmole), 1 mM L-aspartate, 125 μM GTP, 150 μM streptomycin sulfate, 5 mM $MgCl_2$, 50 mM HEPES-NaOH (pH 7.4), and 100 μg of enzyme protein, and was incubated for 20 min. For details, see the text. ●, no IPAMP; ■, 1 mM; ○, 2 mM; ▲, 3 mM; K_m for IMP, 0.1 mM; K_i for IPAMP, 3.0 mM; V_{max}, 5.6 nmoles/min/mg of protein.

assay mixture for stabilization of the enzyme (36).

The conversion of [8-^{14}C]IMP to succinyl-AMP was measured after chromatographic separation in solvent 1 (Table 1). Since synthetic succinyl-AMP was not available as a marker, the reaction product was identified using unlabeled IMP and [U-^{14}C]L-aspartate as substrates. With respect to the chromatographic movement, the product of this reaction was identical with the product obtained from [8-^{14}C]IMP, and its formation was dependent on L-aspartate and GTP; L-glutamate did not substitute for L-aspartate.

IPAMP inhibited the formation of succinyl-AMP in a noncompetitive manner with respect to IMP (Fig. 6B). The inhibi-

tion produced by 1.1 and 2.2 mM IPAMP at 200 μM IMP was also not overcome by increasing the concentration of either GTP or aspartate 10-fold. The K_m for IMP was 91 μM, 3 times that reported for the enzyme in Ehrlich ascites cells (17), and the K_i for IPAMP was 3.0 mM. An IMP analogue, 6 thio-IMP, was also found to inhibit this enzyme in Ehrlich ascites cells in a noncompetitive manner with respect to IMP (34). The nucleoside, IPAdo, at 4 mM had no effect on the conversion of 200 μM IMP to succinyl-AMP.

Adenylosuccinase (adenylosuccinate:AMP lyase, EC 4.3.2.2). A modified method of Carter and Cohen (37) was used for the enzyme assay. Since succinyl-AMP was not available for use as the substrate, the reverse reaction, i.e., the conversion of [8-^{14}C]AMP and fumarate to succinyl-AMP, was examined. In potassium phosphate buffer, pH 7.4, at 125 μM AMP and 1 mM fumarate, only traces of succinyl-AMP were formed in 60 min, even in the presence of large amounts of enzyme (2 mg of protein per 0.4 ml). This appeared to reflect the equilibrium of the adenylosuccinase reaction, which lies far toward the formation of AMP (37). When the concentration of fumarate was increased to 25 mM, the formation of succinyl-AMP in the presence of 400 μg of protein per 0.4 ml was linear for at least 10 min. Under these conditions 3.2 and 6.4 mM IPAMP inhibited the formation of succinyl-AMP by 23 and 54%, respectively.

5-Phosphoribosyl pyrophosphate synthetase (ATP:D-ribose 5-phosphotransferase, EC 2.7.1.15). The enzyme was assayed as described by Wong and Murray (38). The conversion of 1 mM [G-^{14}C]ribose 5-phosphate (0.125 μCi/μmole) to PRPP by the S-180 cell extract (200 μg of protein per 0.4 ml) was dependent on ATP (2.5 mM) and was linear for at least 30 min when carried in 63 mM potassium phosphate, pH 7.4, in the presence of 5 mM $MgCl_2$ and 12.5 mM glutathione. In the presence of [8-^{14}C]adenine and unlabeled ribose 5-phosphate labeled AMP was formed, providing additional evidence that PRPP had indeed been synthesized. PRPP synthetase activity in S-180 cell extracts was 2.4 nmoles/min/mg of protein. The formation of PRPP was inhibited by 14, 39, and 65% at 1, 2.5, and 5 mM IPAMP, respectively; duplicates varied less than ±10%.

Guanylate kinase (ATP:GMP phosphotransferase, EC 2.7.4.8). This enzyme was assayed by a modified method of Miech *et al.* (39). A clear chromatographic separation of the products, GDP and GTP, from the substrate, GMP, was achieved by using solvent 2. The phosphorylation of GMP was dependent on ATP. In 63 mM potassium phosphate, pH 7.4, containing 5 mM $MgCl_2$, 2.5 mM ATP, 125 μM GMP(2 μCi/μmole), and 21 μg of protein per 0.4 ml, the reaction was linear for at least 20 min. The GMP kinase activity of the extract was 26 nmoles/mg of protein per minute. Under these conditions 8 mM IPAMP did not significantly inhibit the reaction. Lowering the concentration of ATP to 125 μM produced approximately 20% inhibition by 6.2 mM IPAMP.

DISCUSSION

The purpose of the present study was to explore the site of action responsible for the cytotoxicity of IPAdo. Several pieces of evidence indicated that IPAMP, and not IPAdo, was responsible for the cytotoxicity. In fact, it had been shown earlier that drug-induced resistance to IPAdo in S-180 cells was associated with a loss of adenosine kinase (5); this led to a drastic reduction in IPAMP levels in resistant cells. The present studies demonstrate that at growth-inhibitory concentrations of IPAdo (20–100 μM), IPAMP accumulated at millimolar levels in the cell water. At the same time the intracellular IPAdo concentration was equal to that outside. In fact, IPAdo comprised only 2% of the cellular antimetabolite in sensitive cells, but 20% in resistant cells (5). The disappearance of IPAMP was found to be rapid in all cells studied so far ($t_{1/2}$ = 37 ± 7 min), but the breakdown into inactive free base was more rapid in cells naturally resistant to the analogue (2). This again resulted in lower cellular levels of IPAMP in the resistant cells. The present study demonstrates that several enzymes involved in the biosynthesis of purine nucleotides were indeed inhibited by IPAMP, but not by IPAdo.

Some general indication for the site of

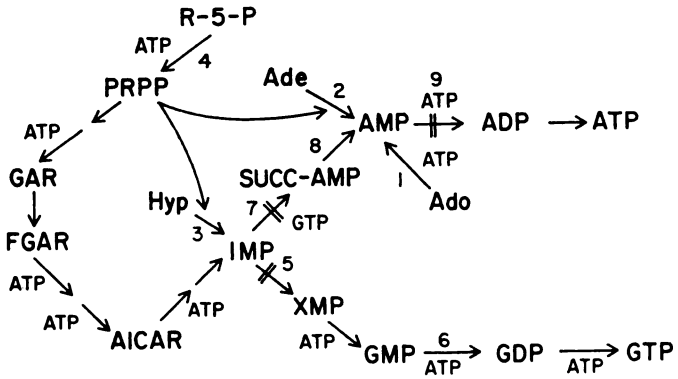

FIG. 7. *De novo and salvage pathways for synthesis of purine nucleotides*

The numbers indicate the reactions as listed in Table 3, and the double cross bars, the reactions most likely affected by the IPAMP formed within the cells from IPAdo. R-5-P, ribose 5-phosphate; GAR, glycinamide ribonucleotide; FGAR, formylglycinamide ribonucleotide; AICAR, 5-amino-4-imidazolecarboxamide ribonucleotide.

action of IPAMP was given by the observation that growth inhibition was unaffected by preformed purines provided in the medium. This suggested that a site other than one concerned with the early steps of purine biosynthesis was involved. Yet, as reported here, a 50% growth-inhibitory concentration of IPAdo caused about 40% inhibition of the cellular formation of formylglycinamide ribonucleotide. It was therefore necessary to consider the possibility that IPAdo may have interfered with the utilization of the preformed purines. It had been shown earlier that IPAdo was a potent inhibitor of the uptake of adenosine (K_i, 1.4 μM) and of other nucleosides as well (8, 9). Thus lack of protection by adenosine, when used at normal levels, was not surprising. IPAMP, on the other hand, was found to inhibit adenine phosphoribosyltransferase. This may have restricted the utilization of adenine, thereby explaining the lack of protection by this purine. In contrast, hypoxanthine phosphoribosyltransferase was only insignificantly inhibited by IPAMP. Thus hypoxanthine was one purine which should have provided protection, assuming that the site of action concerned the early steps of purine biosynthesis. The lack of protection by hypoxanthine that was observed strengthened the hypothesis that the critical site of action of IPAMP was other than an early step in purine biosynthesis.

The effects of IPAMP on a number of enzymes concerned with purine metabolism are summarized in Table 3 and Fig. 7. Based solely on K_i values, IPAMP would have to be considered a poor inhibitor of all these enzymes. However, the intracellular levels of IPAMP, as shown in this study, were very high when compared with the prevailing levels of AMP and IMP; comparison of the intracellular $[I]/[S]$ ratio with the K_i/K_m ratio leads to the conclusion that AMP kinase and inosinate dehydrogenase may well be the critical sites of action of IPAMP.

In the S-180 cell system IPAdo led to significant reduction in ATP pools while AMP accumulated. The reduction of ATP pools could be the result of the inhibition of AMP kinase by IPAMP. Also, since IPAMP accumulates in the cells at millimolar levels (in spite of its rapid breakdown), one must conclude that new IPAMP was being synthesized continuously; the use of ATP for this synthesis may have contributed to the reduction in ATP levels in cells exposed to IPAdo. It is also of interest that IPAdo caused a 50% decrease in ATP pools of rat liver *in vivo*.[3] The observed inhibition of formylglycinamide ribonucleotide synthesis (Figs. 1 and 7) in intact S-180 cells exposed to IPAdo may indeed reflect the reduced ATP levels. While Nelson and Parks (42), on treatment of S-180 cells with 6-methyl-

[3] Y. Rustum, unpublished observations.

TABLE 3

IPAMP as inhibitor of enzymes of purine metabolism

Enzyme	Inhibition	K_i/K_m	Intracellular[a]	
			[S]	[I]/[S]
			mM	
Salvage				
1. Adenosine kinase	Competitive vs. ATP	1.4	4.3	0.5
2. Adenine PRPP transferase	Competitive vs. PRPP	4.0	4.6[b], 0.01[c]	0.4, 200
3. Hypoxanthine PRPP transferase	Poor			
De Novo				
4. PRPP synthetase	Moderate			
5. IMP dehydrogenase	Competitive vs. IMP	15	$\ll 0.1$	$\gg 20$
6. GMP kinase	Insignificant			
7. Adenylosuccinate synthetase	Noncompetitive vs. IMP	K_i, 3.0 mM	$\ll 0.1$	
8. Adenylosuccinate lyase	Poor			
9. AMP kinase	Competitive vs. AMP	8.8	0.04	50

[a] [S] indicates the concentration of the competing substrate in cell water under normal conditions. [I], the intracellular concentration of IPAMP, is taken as 2 mM, corresponding to 100% growth-inhibitory conditions (see Figs. 1 and 2).

[b] This value for PRPP was derived from the data of Henderson and Khoo (40) for S-180 ascites cells incubated with 5.5 mM glucose, assuming that the wet cell pellet contained 49% of cell water as in the case of S-180 cells grown in culture (23).

[c] Refers to the value of Kelley *et al.* (41) for human fibroblasts in culture.

thioinosine, also observed a 50% reduction in ATP and GTP pools, it is of interest that Warnick and Paterson (43), using L-5178Y cells, found a 40% reduction of these pools even when cell proliferation was unaffected. This suggests that a 40% reduction of nucleotide levels may not be critical for cell growth.

This study has shown that the cytotoxicity of IPAdo requires high intracellular levels of IPAMP. These levels are restricted by the reduction in ATP, which is required for 5′-IPAMP formation; second, as shown here, IPAMP inhibits adenosine kinase, and thus its own formation, by competing with ATP; third, the intracellular breakdown of IPAMP is very rapid ($t_{1/2}$ = 37 min) in all cells studied so far (2) and thus requires the constant presence of IPAdo. Whether a new adenosine analogue can be designed, which can resist such breakdown, deserves to be explored. IPAdo represents an interesting and unusual example of a cytotoxic agent having numerous sites of action which either singly or in concert are responsible for cytotoxicity. Since substrate pools and enzyme activities in different tissues are likely to differ, the multiplicity of the sites of action may well constitute a basis for selectivity of action which has been observed in different tissues *in vivo* (44, 45).

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