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HYPOXANTHINE PHOSPHORIBOSYLTRANSFERASE AND GUANINE METABOLISM OF ADENOCARCINOMA 755 CELLS*

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Abstract—Hypoxanthine phosphoribosyltransferase (IMP: pyrophosphate phosphoribosyltransferase, EC 2.4.2.8), purified 60- to 160-fold from an acetone powder of Adenocarcinoma 755 cells, catalyzes the production of nucleotides from several purine bases and base analogs. Michaelis constants for hypoxanthine, guanine, 6-mercaptopurine and 6-thioguanine are 4·0, 2·7, 10 and 10 μM respectively. The Michaelis constant for 5-phosphoribosyl 1-pyrophosphate varies with the purine base used as substrate. Some purine base analogs inhibit the isolated enzyme, but nucleotides and nucleotide analogs are more potent, as judged by inhibition constant/Michaelis constant ratios. This indicates strong endproduct control of enzyme activity.

6-Thioguanine, 6-mercaptopurine and 1-benzyl-6-mercaptopurine inhibit the incorporation of guanine and hypoxanthine, but not of adenine, into the nucleic acids of intact cells. 6-Methylthiopurine ribonucleoside, an inhibitor of *de novo* purine biosynthesis, reduces the incorporation of all three bases. Identification of the metabolites of labeled guanine, hypoxanthine and adenine leads to the conclusion that a major site of inhibition of purine base metabolism by the nucleotides of 6-thioguanine and 6-mercaptopurine is at hypoxanthine phosphoribosyltransferase.

THE PURINE bases, guanine and hypoxanthine, and several purine analogs are converted to the ribonucleotide derivatives by hypoxanthine phosphoribosyltransferase (IMP: pyrophosphate phosphoribosyltransferase, EC 2.4.2.8). For guanine and hypoxanthine, this represents a salvage pathway for nucleotide production. The effectiveness of the purine analogs, 6-mercaptopurine and 6-thioguanine, in inhibiting cell growth is dependent on their transformation to the ribonucleotide by this enzyme.[1, 2] Previous publications report the partial purification of hypoxanthine phosphoribosyltransferase from *Escherichia coli*,[3] beef liver,[4] Ehrlich ascites tumor cells,[5] *Salmonella typhimurium*,[6] yeast,[7] and human erythrocytes.[8] Interest in this enzyme has been greater since the observation that human mutants lacking hypoxanthine phosphoribosyltransferase activity have a severe neurological disease.[9, 10]

This report concerns an extensive purification of the enzyme from Adenocarcinoma 755 cells, an investigation of its kinetic properties, and an evaluation of a number of potential inhibitors, both for the isolated enzyme and for purine metabolism in intact cells.

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MATERIALS AND METHODS

Dr. John A. Montgomery of the Organic Chemistry Division of Southern Research Institute generously supplied the following: 6-mercaptopurine, 6-mercaptopurine-^{35}S, 6-thioguanine, 1-benzyl-6-mercaptopurine, 6-thioGMP, 8-azaGMP, 6-mercaptopurine ribonucleotide and most of the analogs that were less effective as inhibitors of hypoxanthine phosphoribosyltransferase. Isoguanine sulfate was obtained from Mann Research Laboratories and 2, 6-dimercaptopurine from Francis Earle Laboratories, Peekshill, N.Y. Unlabeled bases, nucleotides and 5-phosphoribosyl 1-pyrophosphate (PP-ribose-P) were purchased from P-L Biochemicals. Guanine-8-^{14}C (37·5 mc/m-mole) and hypoxanthine-8-^{14}C (50 mc/m-mole) were purchased from Schwarz Bioresearch and adenine-8-^{14}C (12·2 mc/m-mole) from New England Nuclear Corp. Snake venom (*Crotalus atrox*) was supplied by Ross Allen's Reptile Institute, Silver Springs, Fl.

Adenocarcinoma 755 cells, grown in swirl cultures as described by Kelley *et al.*,[11] were harvested by centrifugation and washed twice in 0·9% NaCl. An acetone powder was prepared from the freshly harvested cells.

Two types of assays for phosphoribosyltransferase activity were used in these studies. Assay A was a spectrophotometric one which used a Gilford 2000 recording spectrophotometer. With guanine as the substrate, the increase in absorbance at 257·5 mμ was used; the difference in extinction coefficients for guanine and GMP at this wavelength and at pH 9·25 was 4100. The increase in absorbance at 245 mμ was used with hypoxanthine as substrate; in this case, the difference in extinction coefficients for the base and IMP was 2500. The increase at 255 mμ was used to assay the conversion of 6-thioguanine to the nucleotide; the difference in extinction coefficients was 5100. The standard assay A consisted of: 10 nmoles guanine or 25 nmoles hypoxanthine or 6-thioguanine; 60 μmoles sodium glycinate, pH 9·25; 5 μmoles $MgCl_2$; 250 nmoles PP-ribose-P; and enzyme in a final volume of 1 ml. The enzyme was added last.

Assay B involved the use of radioactive bases as substrates. This type of assay was used when no suitable spectrophotometric assay could be devised, as was the case when 6-mercaptopurine was used and when inhibitors produced absorbance changes in the absence of substrate. Standard assay B consisted of : 5 nmoles 6-mercaptopurine or 2 nmoles hypoxanthine or guanine; 10 μmoles sodium glycinate, pH 9·25; 1 μmole $MgCl_2$; 50 nmoles PP-ribose-P; and enzyme in a total volume of 200 μl. The reaction was stopped after 20 min by adding 50 μl of 4N formic acid and the entire reaction mixture was streaked on a paper strip for chromatography in a solvent of *n*-butyl alcohol and propionic acid.[12] The strips were scanned and the radioactivity of the substrate and product was measured by a Packard 7201 radiochromatogram scanner.

Initial velocities (v) and substrate concentrations ([S]) from the kinetic experiments were fitted by an IBM 1132 computer to the equation, $v = V[S]/K_m + [S]$, where V = maximum velocity and K_m = Michaelis constant. The Fortran program developed by Cleland[13] was used. From this procedure, values for V, $1/V$, K_m and K_m/V were obtained.

For incorporation experiments using intact cells, 100 ml cells in swirl culture were used. The radioactive precursor (10 μc) and potential inhibitor were added simultaneously. After 2 hr, the cells were harvested by centrifugation, washed once with 0·9% NaCl, and poured into 40 ml of boiling ethyl alcohol. Soluble materials were extracted by boiling for 10 min. The residue was removed by centrifuging at 27,000 g for 10 min and the solution was evaporated with reduced pressure at 40° to about 5 ml. This

material was lyophilized to dryness and dissolved in water, with 0·8 ml being added for each 10^8 cells present. A portion (60 μl) of the solution was spotted on paper for two-dimensional chromatography. The solvents were 70% phenol and the *n*-butyl alcohol–propionic acid system mentioned above. Metabolites were located by autoradiography and were cut from the chromatogram either for counting in a liquid scintillation spectrometer or for elution and identification. The identification process involved several procedures: (1) all of the radioactive metabolites were treated with HCl (1 N, 1 hr at 100°) to hydrolyze all of the metabolites to purine bases and were then subjected to chromatography with water-saturated *n*-butyl alcohol–NH_3 (100:1, v/v) along with the appropriate base standards; (2) compounds migrating in the two-dimensional system as bases and ribonucleosides were rechromatographed, with standards, in the *n*-butyl alcohol–propionic acid solvent; (3) compounds migrating as nucleotides were rechromatographed in a solvent containing 1 liter of 0·1 M sodium phosphate (pH 6·8), 600 g $(NH_4)_2SO_4$ and 20 ml *n*–propyl alcohol; (4) the ribonucleotides were also treated with crude snake venom and the resulting ribonucleosides were characterized by chromatography in *n*-butyl alcohol–acetic acid–water (50:20:30, v/v).

From the residue remaining after the ethyl alcohol extraction, sodium nucleates were isolated by extraction with hot 10% NaCl, precipitated with ethyl alcohol, and assayed for radioactivity. The nucleates were treated overnight with 1 N KOH at room temperature, the solution was neutralized with $HClO_4$, and $KClO_4$ was removed by centrifugation. The soluble portion was acidified to pH 4 with acetic acid, a few drops of 1 M $MgCl_2$ were added, and ethyl alcohol was added to precipitate the DNA, which was also assayed for radioactivity.

RESULTS

Purification. The procedure for purifying hypoxanthine phosphoribosyltransferase from Adenocarcinoma 755 cells is a rapid and simple one. I extract an acetone powder of cultured cells with 33 mM potassium phosphate buffer, pH 7·0, centrifuge at 22,000*g* and place the extract in a water bath at 65° for 8 min. I remove the precipitated protein, place the extract on a DEAE-cellulose column (1 × 15 cm) previously equilibrated at pH 7·0 with potassium phosphate, and elute with a 0 to 0·2 M linear gradient of the buffer. The most active fractions have a specific activity of 300–800 nmoles/min/mg, and the enzyme is 60- to 160-fold purified.

pH optimum. The effects of pH and of buffer on the velocity of the reactions are interesting (Fig. 1). The optimum pH with guanine as the substrate is lower in the presence of either Tris chloride or sodium bicarbonate buffer than with sodium glycinate. Such effects are not noted when hypoxanthine is the substrate. The velocities with guanine and with hypoxanthine are maximal and almost equivalent in sodium glycinate at pH 9·25. This buffer is present in all of the kinetic assays.

Substrates. A number of purine bases serve as substrates for hypoxanthine phosphoribosyltransferase. In each case, when double-reciprocal plots are made varying either the purine base or the PP-ribose-P concentration, parallel lines are produced. Those for guanine and PP-ribose-P are in Fig. 2. Replotting the reciprocals of the maximum velocities with the reciprocals of the substrate concentrations enables one to determine the Michaelis constant (K_m) for each substrate. The value for guanine is 2·7 μM and that for PP-ribose-P is 115 μM. Substitution of hypoxanthine, 6-mercaptopurine or 6-thioguanine for guanine in similar assays gives the same type of plot.

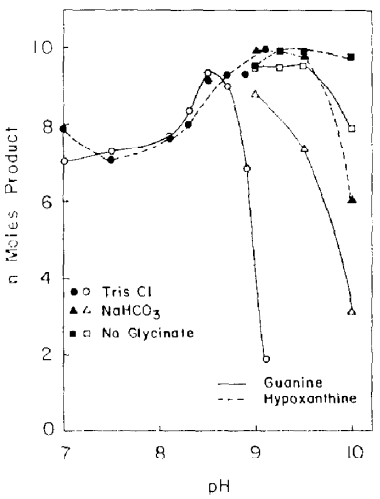

FIG. 1. Effect of buffer and pH on the activity of hypoxanthine phosphoribosyltransferase. Assay B was used with the modifications that the guanine and hypoxanthine concentrations were increased to 200 μM and that the buffer specified was present. Substrates were adjusted to the pH of the assay.

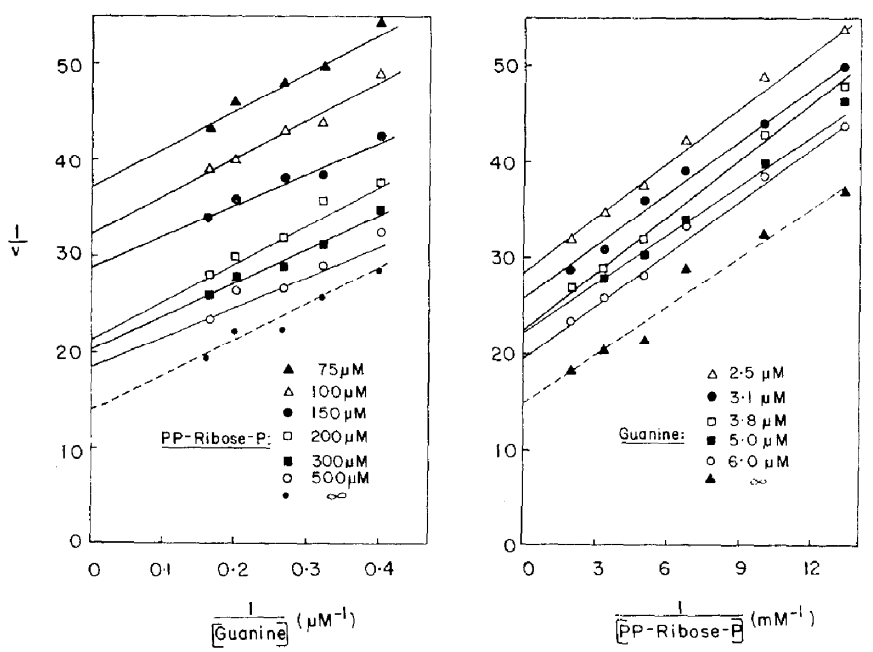

FIG. 2. Kinetics of guanine and PP-ribose-P as substrates of hypoxanthine phosphoribosyltransferase. Assay A was used. The dashed lines represent secondary plots of the maximum velocities. Values for the vertical axis are reciprocals of the change in absorbance during the intitial 12 min of the reaction.

Table 1 summarizes the kinetic values for substrates of the enzyme. Isoguanine, 8-azaguanine, 3-deaza-6-mercaptopurine, 2,6-dimercaptopurine and 4-hydroxy-6-mercaptopyrazolo (3, 4-d) pyrimidine show no detectable reaction as substrates, as determined by the fact that there is no appreciable change in the spectrum of these compounds on prolonged incubation in the presence of the components of the assay system.

TABLE 1. MICHAELIS CONSTANTS FOR SUBSTRATES OF HYPOXANTHINE PHOSPHORIBOSYLTRANSFERASE

Varied substrate	K_m for varied substrate (μM)	Fixed substrate
Guanine	2·7	PP-ribose-P
PP-ribose-P	115	Guanine
Hypoxanthine	4·0	PP-ribose-P
PP-ribose-P	36	Hypoxanthine
6-Mercaptopurine	10*	PP-ribose-P
PP-ribose-P	38*	6-Mercaptorpurine
6-Thioguanine	10	PP-ribose-P
PP-ribose-P	80	6-Thioguanine

*Assay B was used to determine these values.

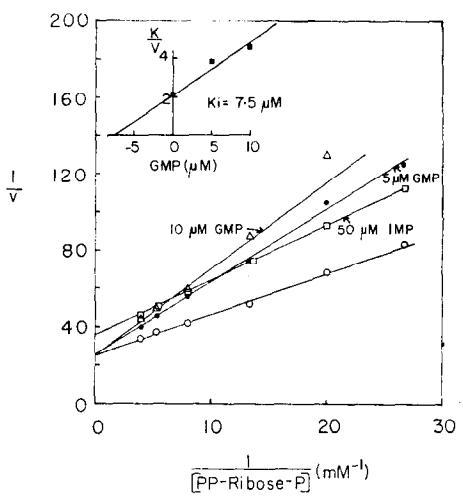

FIG. 3. Inhibition of hypoxanthine phosphoribosyltransferase by GMP and by IMP. Assay A was used, with guanine at a fixed concentration. The inset is a plot of the slopes of the lines obtained with GMP as inhibitor. It was used to determine the value for K_i. Values for the vertical axis are the same as for Fig. 2.

Inhibitors of the isolated enzyme. GMP, an endproduct of the reaction catalyzed by hypoxanthine phosphoribosyltransferase, shows noncompetitive inhibition with either guanine or hypoxanthine, but it is competitive with PP-ribose-P in the presence of either base. The inhibition constant (K_i) for GMP with guanine as the second substrate (at fixed concentration) is 7·5 μM (Fig. 3). The same value is obtained when hypoxanthine is the second substrate. The inhibition by other nucleotides is also non-

competitive with guanine or hypoxanthine, but most show competitive inhibition with PP-ribose-P. IMP (Fig. 3) and 6-thioGMP (Fig. 4) are the exceptions. IMP is noncompetitive with either substrate, but the deviation by 6-thioGMP from competitive inhibition with PP-ribose-P is only slight at the lower concentrations tested. The inhibition constants for nucleotide inhibitors are listed in Table 2. GMP, dGMP, 6-thioGMP and 6-mercaptopurine ribonucleotide are especially potent inhibitors.

The purine bases, guanine and hypoxanthine, and the base analogs, isoguanine, 6-mercaptopurine and 6-thioguanine, are competitive inhibitors of the base used as substrate, but they are noncompetitive with PP-ribose-P. Figure 5 shows examples of

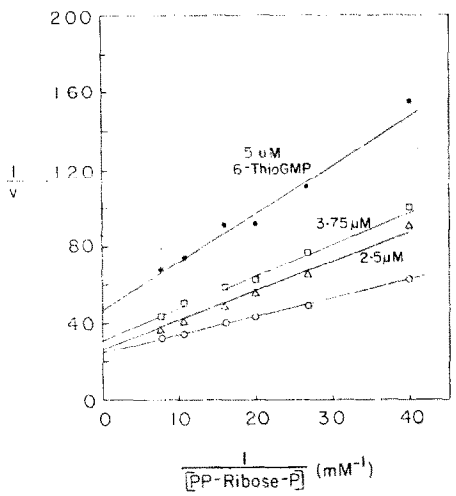

FIG. 4. Inhibition of hypoxanthine phosphoribosyltransferase by 6-thioGMP. Assay A was used, with hypoxanthine at a fixed concentration. Values for the vertical axis are the same as for Fig. 2.

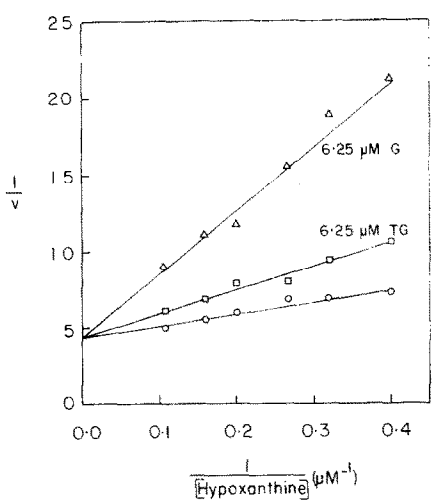

FIG. 5. Inhibition of hypoxanthine phosphoribosyltransferase by guanine and 6-thioguanine. Assay B was used, with PP-ribose-P at a fixed concentration. Values for the vertical axis are reciprocals of the nanomoles of product formed in 20 min. Abbreviations: G, guanine; TG, 6-thioguanine.

TABLE 2. INHIBITION CONSTANTS FOR COMPETITIVE INHIBITORS OF HYPOXANTHINE PHOSPHORIBOSYLTRANSFERASE

Inhibitor	Inhibition constant (varied substrate/fixed substrate) (μM)			
	Guanine/ PP-ribose-P	PP-ribose-P/ guanine	Hypoxanthine/ PP-ribose-P	PP-ribose-P/ hypoxanthine
Guanine		noncompetitive	1·5*	noncompetitive
Hypoxanthine	3·2	noncompetitive		
6-Mercaptopurine	5·0	noncompetitive	5·0	noncompetitive
6-Thioguanine	4·5*		7·0*	noncompetitive
Isoguanine	1·8	noncompetitive	1·6	noncompetitive
6-Mercaptopurine ribonucleoside	not competitive†	not competitive	not competitive	not competitive
2, 6-Dimercaptopurine	not competitive		not competitive	not competitive
GMP	noncompetitive	7·5	noncompetitive	7·5
IMP		noncompetitive	noncompetitive	noncompetitive
dGMP	noncompetitive	2·0		2·5
6-Thio-GMP		not competitive		not competitive
dGDP		24		16
8-AzaGMP	noncompetitive	27		40
6-Mercaptopurine ribonucleotide	noncompetitive	10	noncompetitive	9·0
PP_i	not competitive	230		320
Others†				

*Assay B was used to determine these values.

†Inhibitors showing neither strict competitive nor strict noncompetitive inhibition are listed as "not competitive."

‡Compounds inhibiting more than 15% but less than 60% at 0·1 mM are: 6-thiouric acid, 8-azaguanine, *trans*-2-[9-(mercaptopurinyl)] cyclopentanol, *cis*-2-[9-(mercaptopurinyl)] cyclopentanol, 9-cyclohexyl-6-mercaptopurine, 9-isopropyl-6-mercaptopurine, 9-butyryl-6-mercaptopurine, 9-butyryl-6-mercaptopurine (ethyl ester), 9-cyclopentyl-6-mercaptopurine, 9-*n*-butyl-6-mercaptopurine, 6, 6′-dithiodi (9-*n*-butyl-purine), 2-ethyl-6-mercaptopurine, 2-hydroxy-6-mercaptopurine, 6-mercaptopurine ribonucleoside disulfide, 6-thioguanosine, 9-[β-DL-2α, 3α-dihydroxy-4β-(hydroxy-methyl)cyclopentyl]-9H-purine-6(1H)-thione, 6-chloropurine, 1-methylhypoxanthine, xanthine and 4-hydroxy-6-aminopyrazolo(3, 4-d)pyrimidine.

Compounds inhibiting less than 15% at 0·1 mM are: GDP, ADP, IDP, ATP, GTP, UTP, ITP, dATP, CMP, CTP, dCDP, dCPT, TTP, 5-methyl-dCMP, dAMP, dGTP, 5-fluoro-UMP, AMP, dADP, dUTP, dCMP, CDP, dIMP, TMP, TDP, $NADPH_2$, $NADH_2$, $NADP^+$ NAD^+, UMP, 6-aza-UTP, guanosine, deoxyguanosine, adenine, adenosine, inosine, 6-aza-UMP, 5-fluoro-UTP, XMP, 6-methylthiopurine ribonucleotide, 2′- and 3′-AMP, tubercidin monophosphate, formycin B, 8-azainosine, 6-allylthiopurine ribonucleoside, 6-methylthiopurine ribonucleoside, 7-deazainosine, 6-chloropurine ribonucleoside, 6-thioxanthine, 9-ethyl-8-azaguanine, 2-fluorohypoxanthine, 8-azahypoxanthine, 9-ethylhypoxanthine, 9-benzylhypoxanthine, 9-*n*-butylhypoxanthine, 9-cyclohexylhypoxanthine, 9-butyrylhypoxanthine, 9-*n*-butyl-6-sulfonylpurine, 1-methyl-6-mercaptopurine ribonucleoside, 3- hydroxymethylcyclopentyl hypoxanthine, uric acid, 4-hydroxypyrazolo(3, 4-*d*)pyrimidine, caffeine, 4-amino-6-hydroxypyrazolo(3, 4-*d*) pyrimidine, 6-mercaptopyrazolo(3, 4-*d*)pyrimidine, 4, 6-dihydroxypyrazolo(3, 4-*d*)pyrimidine, 9-[β-DL-2α, 3α-dihydroxy-4β-(hydroxymethyl)cyclopentyl]adenine, 2-fluoro-6-mercaptopurine ribonucleoside, 1-benzylhypoxanthine, 1-benzyl-6-mercaptopurine, 2-chloro-7-ethylhypoxanthine, 7-ethylhypoxanthine, 7-cyclopentyl-6-mercaptopurine, 1-cyclopentylhypoxanthine, 1-cyclopentyl-6-mercaptopurine, 1-(2′-azidoethyl)-hypoxanthine, 1-(2′-aminoethyl) hypoxanthine, 9-[β-DL-2α, 3α-dihydroxy-4β-(hydroxymethyl)cyclopentyl] hypoxanthine, and 9-[β-DL-2α, 3α-dihydroxy-4β-hydroxymethyl)-cyclopentyl]-6-methylthio-9H-purine.

TABLE 3. EFFECT OF INHIBITORS ON THE METABOLISM OF ^{14}C-ADENINE, HYPOXANTHINE AND GUANINE

Inhibitor	Conc. of inhibitor (mM)	Sp. act. of combined nucleic acids (pc/μg)	Sp. act. of DNA (pc/μg)	Total alcohol-soluble (dpm)	Adenine (% of total)	Hypoxanthine (% of total)	Guanine (% of total)	Adenosine (% of total)	Inosine (% of total)	Xanthosine (?) (% of total)	NAD (% of total)	Unidentified (% of total)	AMP (% of total)	IMP + GMP (% of total)	Succinyl-AMP (?) (% of total)	ADP (% of total)	ATP + GDP + GTP (% of total)
Experiment 1: ^{14}C-adenine as precursor																	
None		211	122	405,000	1·8			7·3	1·2	0·1	2·9	0·3	25·3	13·5*	1·9	32·2	13·5*
6-Thioguanine	0·1	229	144	285,000	2·2			6·7	1·1	0·1	3·3	0·3	24·8	10·7	2·3	34·4	14·0
6-Methylthiopurine ribonucleoside	0·05	156	99	349,000	2·6			17·7	4·8	0·2	2·5	0·2	20·6	19·6	1·6	22·0	8·0
1-Benzyl-6-mercapto-purine	0·1	207	116	337,000	2·0			7·8	1·4	0·1	3·0	0·2	43·8	12·5	2·8	21·1	5·3
6-Mercaptopurine	0·1	248	164	352,000	1·8			15·6	4·0	0·1	3·7	0·3	26·4	28·7	1·7	14·9	2·7
Experiment 2: ^{14}C-hypoxanthine as precursor																	
None		243	434	207,000	2·0	3·8	0·5		0·5	0·1	3·0	0·3	24·3	14·7*	1·3	30·9	18·4*
6-Thioguanine	0·1	24	40	21,000	1·6	10·0	1·2		1·0	0·1	2·3	0·2	28·2	18·9	1·1	25·1	10·3
6-Methylthiopurine ribonucleoside	0·05	111	184	116,000	4·5	4·8	0·2		0·9	0·2	3·4	0·4	30·0	12·6	0·7	32·2	10·2
1-Benzyl-6-mercapto-purine	0·1	140	148	141,000	1·7	7·2	0·3		1·6	0·1	2·5	0·3	23·7	38·1	0·7	17·8	5·9
6-Mercaptopurine	0·1	102	145	100,000	2·7	12·0	0·2		3·0	0·1	4·1	0·5	22·1	25·9	1·0	22·3	6·2
Experiment 3: ^{14}C-guanine as precursor																	
None		116	309	7090			29·0						4·2	29·9†			36·9†
6-Thioguanine	0·1	41	108	2430			34·2						7·6	29·1			29·0
6-Methylthiopurine ribonucleoside	0·05	53	144	3490			51 6						5·1	20·2			23·0
1-Benzyl-6-mercapto-purine	0·1	58	148	6210			25·0						4·1	44·2			26·6
6-Mercaptopurine	0·1	57	98	3058			42·3						8·7	24·9			24·7

*Only small amounts of GMP, GDP and GTP are present when ^{14}C-adenine or ^{14}C-hypoxanthine is the labeled precursor.
†Only small amounts of IMP and ATP are present when ^{14}C-guanine is the labeled precursor.

this type of inhibition, and a summary is in Table 2. With guanine, hypoxanthine or PP-ribose-P as the varied substrate, 2, 6-dimercaptopurine and 6-mercaptopurine ribonucleoside are neither competitive nor noncompetitive at the concentrations tested (5 and 20 μM). A number of other compounds are not potent inhibitors of the enzyme. These are listed at the bottom of Table 2. For base analogs in this list, derivatives of 6-mercaptopurine with substituents in either the 9- or 2-position or in both are moderately inhibitory. With the exception of 8-azaguanine, 6-chloropurine and 1-methylhypoxanthine, all other substitutions result in complete or nearly complete inactivity.

Studies with intact cells. Kinetic investigations with the isolated enzyme provide a list of analogs which might be inhibitors in the intact cell. This list is not exclusive, however, since other analogs could be converted by enzymes in the cell to active inhibitors. Of 35 possible inhibitors tested for inhibition of guanine-8-^{14}C utilization by cells in culture, nine are effective. Four of these are included in Table 3, which shows their effects on guanine-8-^{14}C, adenine-8-^{14}C, and hypoxanthine-8-^{14}C utilization. Of these four, 6-thioguanine and 6-mercaptopurine (and their ribonucleotides) are inhibitors of the isolated enzyme. 6-Methylthiopurine ribonucleoside (and its ribonucleotide) and 1-benzyl-6-mercaptopurine are not inhibitors.

The uptake of labeled adenine, hypoxanthine and guanine into alcohol-soluble materials is reduced in the presence of any of the four analogs (Table 3), but the analogs are generally less effective against adenine uptake than against hypoxanthine and guanine uptake. 1-Benzyl-6-mercaptopurine does not greatly reduce the uptake of guanine, hypoxanthine or adenine, but the presence of the analog gives rise to an accumulation of the ribonucleotide of the base used as precursor. The inhibitors generally lead to small increases (expressed as per cent of total radioactivity on the chromatogram) in the size of the pools of the radioactive base used; but, with one exception, the amounts of di- and triphosphates, considered together, are reduced by the inhibitors. The exception is that 6-thioguanine does not reduce the percentage of ADP and ATP produced from labeled adenine. Guanine uptake into alcohol-soluble materials is low. This may reflect small pool sizes for the soluble metabolites of this base.

None of the four analogs shows a strong selective effect on RNA or DNA metabolism. In no case is there a great difference between the percentage inhibition of the incorporation into the combined nucleic acids and into the DNA. The incorporation of adenine-8-^{14}C into nucleic acids is moderately stimulated by 6-mercaptopurine. A similar though less striking effect is seen with 6-thioguanine.

The most potent inhibition of incorporation of adenine-8-^{14}C into nucleic acids (25 per cent) is by 6-methylthiopurine ribonucleoside. This inhibitor shows no base specificity for inhibition of the incorporation into nucleic acids; hypoxanthine-8-^{14}C and guanine-8-^{14}C incorporation are also inhibited. In contrast to this effect of 6-methylthiopurine ribonucleoside, the other three inhibitors are specific in that they inhibit the incorporation of hypoxanthine and guanine, but not of adenine (Table 3).

In addition to the inhibitors listed in Table 3, guanosine, deoxyguanosine, adenine, hypoxanthine and 1-benzylhypoxanthine at 0·1 mM concentrations also decrease incorporation of guanine into nucleic acids. Guanosine, which inhibits 75 per cent, is apparently effective by diluting the labeled guanine after cleavage to the base. Deoxyguanosine, also with 75 per cent inhibition, may act similarly but it has the

additional effect of greatly reducing the production of di- and triphosphates. Adenine inhibits 65 per cent and is likely to be effective by competing with guanine for PP-ribose-P. Hypoxanthine, with 63 per cent inhibition, competes with guanine for hypoxanthine phosphoribosyltransferase. 1-Benzyl-hypoxanthine inhibits 30 per cent; its mechanism of action is a mystery.

Compounds which have little effect on the incorporation of guanine into the alcohol-soluble fraction or the nucleic acids are the following: 3-deaza-6-mercaptopurine, 4-hydroxy-6-mercaptopyrazolo (3, 4-*d*) pyrimidine, 2, 6-dimercaptopurine, 4-mercapto-pyrazolo (3, 4-*d*) pyrimidine, 1-cyclopentyl-6-mercaptopurine, 2-hydroxy-6-mercapto-purine, 9-isopropyl-6-mercaptopurine, 7-cyclopentyl-6-mercaptopurine, 2-fluoro-6-mercaptopurine ribonucleoside, 1-methyl-6-mercaptopurine ribonucleoside, 6-thiouric acid, isoguanine, 8-azaguanine, 4-hydroxy-6-aminopyrazolo (3, 4-*d*) pyrimidine, uric acid, 4-hydroxypyrazolo (3, 4-*d*) pyrimidine, 8-azahypoxanthine, 9-ethylhypoxanthine, 1-methylhypoxanthine, 2-chloro-7-ethylhypoxanthine, 7-ethylhypoxanthine, 1-cyclopentylhypoxanthine, 1-(2′-azidoethyl)hypoxanthine, 1-(2′-aminoethyl) hypoxanthine, 6-chloropurine and caffeine.

DISCUSSION

Purification of hypoxanthine phosphoribosyltransferase from Adenocarcinoma 755 cells to a high specific activity and investigation of the kinetic properties of the purified enzyme indicate that only one enzyme is involved in converting guanine, hypoxanthine, 6-mercaptopurine and 6-thioguanine to the corresponding nucleotides. This is in accord with studies on this enzyme from yeast[7] and from human erythrocytes,[8, 14–17] but contrasts with the contention that phosphoribosyltransferase activity for guanine and for hypoxanthine can be separated.[5] The specific activity of the enzyme extracted from Adenocarcinoma 755 cells is higher than any previously reported for a mammalian source.

The K_m values for substrates of hypoxanthine phosphoribosyltransferase do not differ greatly from those for Ehrlich ascites tumor cells,[5, 18, 19] for yeast[7, 20] and for human erythrocytes.[8, 15] Also, reported values for the K_i for GMP are in the same range as that for the Adenocarcinoma 755 enzyme.[15, 17, 19, 20] Inhibition by the bases and base analogs, adenine, 1-methylhypoxanthine, xanthine, 6-thioguanine, 6-mercapto-purine, 6-chloropurine, 6-amino-4-hydroxypyrazolo(3, 4-*d*)pyrimidine and 8-azaguanine, can be compared to that observed by Krenitsky *et al.*[8] for the enzyme from erythrocytes. The results are very similar, with the exception that 6-chloropurine moderately inhibits the tumor cell enzyme but not that from erythrocytes.

The fact that 8-azaguanine is not noticeably affected by hypoxanthine phosphoribosyltransferase of Adenocarcinoma 755 cells is surprising. 8-Azaguanine is converted to the nucleotide by this enzyme from beef liver,[21] from yeast,[20] from *Salmonella typhimurium*,[6] from *Streptococcus faecalis*[22] and from L1210 lymphomatous tumor;[23] but with enzymes from these sources, the rate of reaction with 8-azaguanine as substrate is much lower than that for guanine. If 8-azaGMP is formed by the tumor cell enzyme, the rate is too slow to detect.

A notable difference between the enzyme from Adenocarcinoma 755 and that from erythrocytes involves inhibition by IMP. The tumor cell enzyme shows noncompetitive inhibition between PP-ribose-P and IMP. The unpurified enzyme from erythrocytes displays strict competitive inhibition, [5] and inhibition of the purified enzyme from this

source becomes competitive at high concentrations of PP-ribose-P.[17] The noncompetitive nature of IMP inhibition of the tumor cell enzyme apparently means that IMP binds less strongly to the active site than to another site on the enzyme. 6-Thio-GMP, to a small extent, may also bind to this site. Another difference in results concerns inhibition by PP_i. Our results show that inhibition by PP_i is competitive with PP-ribose-P in the presence of excess Mg^{2+}. For the unpurified erythrocyte enzyme, inhibition is noncompetitive;[15] for the purified erythrocyte enzyme, inhibition is overcome at high concentrations of Mg^{2+}.[17]

The studies reported here give some rational basis for selecting inhibitors of the salvage pathway for guanine and hypoxanthine. With the isolated enzyme, the base analogs, 6-mercaptopurine, 6-thioguanine and isoguanine, have low K_i values, but when these are divided by the K_m for guanine, the resulting K_i/K_m ratios are high (0·7 to 1·8), an indication of only moderate inhibition. On the other hand, the K_i values for the nucleotides GMP and dGMP are also low, but when divided by the K_m for PP-ribose-P, with which they are competitive, the resulting K_i/K_m ratios are low (0·017 and 0·065), implying strong inhibition. Isoguanine, a competitive inhibitor of guanine and hypoxanthine, does not inhibit the incorporation of guanine into nucleic acids. Apparently, free bases are not strong inhibitors in the intact cell, and nucleotide formation is necessary for activity on the salvage pathway. The potent inhibition by endproducts and analogs of the endproducts shows that the enzyme is under stringent endproduct control. This conclusion differs from that by Atkinson and Murray,[5] and by Murray,[19] who reason that 6-mercaptopurine could be a powerful inhibitor of hypoxanthine phosphoribosyltransferase and that the inhibition by nucleoside monophosphates is relatively unimportant.

From these studies with intact cells, it is evident that only small amounts of purine bases are taken up without subsequent nucleotide production, since the pools of free base in the cells are small. Purines gain entry to mammalian cells by diffusion rather than by active transport,[24] and uptake is greatly dependent on nucleotide production. One may conclude that inhibition of hypoxanthine phosphoribosyltransferase by nucleotide analogs prevents nucleotide production, and such inhibition reduces the uptake of labeled guanine and hypoxanthine.

The stimulation of the incorporation of adenine-8-^{14}C into nucleic acids by 6-mercaptopurine and 6-thioguanine is interesting. Such stimulation has been noted previously for tumor cells.[25, 26] The mechanism by which this occurs is not understood, but perhaps 6-mercaptopurine and 6-thioguanine or metabolites of these analogs stimulate adenine phosphoribosyltransferase (AMP: pyrophosphate phosphoribosyltransferase, EC 2.4.2.7).

6-Methylthiopurine ribonucleoside is converted by adenosine kinase (ATP: adenosine 5′-phosphotransferase, EC 2.7.1.20) to the nucleotide,[27, 28] which exerts a potent inhibition of the *de novo* purine pathway.[29, 30] The lack of inhibition by 6-methylthiopurine ribonucleoside and ribonucleotide on the isolated enzyme and the nonspecific inhibition by 6-methylthiopurine ribonucleoside of the incorporation of all three purine bases into nucleic acids may mean that this analog, by blocking *de novo* purine nucleotide biosynthesis, lowers the ATP content of the cell to such an extent that production of nucleic acids is reduced.

The property of 1-benzyl-6-mercaptopurine in not strongly inhibiting uptake into the alcohol-soluble fraction, coupled with the accumulation of monophosphates and

decrease of di- and triphosphates in its presence, implies that this analog (or a metabolite of it) inhibits GMP kinase (GMP: ATP phosphotransferase, EC 2.7.4.8) and AMP kinase (AMP: ATP phosphotransferase, EC 2.7.4.3). The lowered amounts of di- and triphosphates in the presence of the other inhibitors may mean that they also have some action on these kinases.

6-Thioguanine inhibits utilization of guanine for production of acid-soluble purines and nucleic acids in intact Ehrlich ascites cells.[25] After conversion to the nucleotide, this analog inhibits the following enzymes involved in purine metabolism: PP-ribose-P amidotransferase (ribosylamine 5-phosphate: pyrophosphate phosphoribosyltransferase (glutamate amidating), EC 2.4.2.14),[30, 31] IMP dehydrogenase (IMP: NAD oxidoreductase, EC 1.2.1.14),[32] and GMP kinase.[32, 33] The strong inhibition of hypoxanthine phosphoribosyltransferase adds another site to the list, but inhibition at this site could not be a primary factor in the inhibition of growth, for deletion of this enzyme is not lethal.[1] It is not known which of the remaining sites on the purine pathway is most critical or if any of these is responsible for the toxic effects of 6-thioguanine.

6-Mercaptopurine ribonucleotide also inhibits several enzymes of the purine pathway, but in this case the primary site of inhibition on this pathway has been tentatively identified as PP-ribose-P amidotransferase.[26, 30, 34] As with 6-thioguanine, the inhibition by 6-mercaptopurine ribonucleotide of hypoxanthine phosphoribosyltransferase could not be a decisive factor in the toxicity of this compound. It is possible that the dual inhibition by these analogs of both *de novo* and salvage-pathway production of GMP and IMP is related to their selective, toxic effect on tumors.

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REFERENCES

1. R. W. BROCKMAN, C. SPARKS, M. S. SIMPSON and H. E. SKIPPER, *Biochem. Pharmac.* **2**, 77 (1959).
2. G. A. LEPAGE and M. JONES, *Cancer Res.* **21**, 642 (1961).
3. C. E. CARTER, *Biochem. Pharmac.* **2**, 105 (1959).
4. L. N. LUKENS and K. A. HERRINGTON, *Biochim. biophys. Acta* **24**, 432 (1957).
5. M. R. ATIKNSON and A. W. MURRAY, *Biochem. J.* **94**, 64 (1965).
6. J. C. ADYE and J. S. GOTS, *Biochim. biophys. Acta* **118**, 344 (1966).
7. R. L. MILLER and A. L. BIEBER, *Biochemistry, N.Y.* **7**, 1420 (1968).
8. T. A. KRENITSKY, R. PAPIOANNOU and G. B. ELION, *J. biol. Chem.* **244**, 1263 (1969)
9. M. LESCH and W. L. NYHAN, *Am. J. Med.* **36**, 561 (1964).
10. J. E. SEEGMILLER, F. M. ROSENBLOOM and W. N. KELLEY, *Science, N.Y.* **155**, 1682 (1967).
11. G. G. KELLEY, M. H. VAIL, D. J. ADAMSON and E. A. PALMER, *Am. J. Hyg.* **73**, 231 (1961).
12. A. A. BENSON, J. A. BASSHAM, M. CALVIN, T. G. GOODALE, V. A. HAAS and W. STEPKA, *J. Am. chem. Soc.* **72**, 1710 (1950).
13. W. W. CLELAND, *Adv. Enzymol.* **29**, 1 (1967)
14. W. N. KELLEY, F. M. ROSENBLOOM, J. F. HENDERSON and J. E. SEEGMILLER, *Proc. natn. Acad. Sci. U.S.A.* **57**, 1735 (1967)
15. J. F. HENDERSON, L. W. BROX, W. N. KELLEY, F. M. ROSENBLOOM and J. E. SEEGMILLER, *J. biol. Chem.* **243**, 2514 (1968).
16. J. F. HENDERSON, *Can. J. Biochem. Physiol.* **47**, 69 (1969).
17. T. A. KRENITSKY and R. PAPAIOANNOU, *J. biol. Chem.* **244**, 1271 (1969).
18. M. R. ATKINSON and A. W. MURRAY, *Biochem. J.* **94**, 71 (1965).
19. A. W. MURRAY, *Biochem. J.* **100**, 671 (1966).

20. R. L. MILLER and A. L. BIEBER, *Biochemistry N.Y.* **8**, 603 (1969).
21. J. L. WAY and R. E. PARKS, JR., *J. biol. Chem.* **231**, 467, (1958).
22. R. W. BROCKMAN, C. S. DEBAVADI, P. STUTTS and D. J. HUTCHINSON, *J. biol Chem.* **236**, 1471 (1961).
23. R. W. BROCKMAN, L. L. BENNETT, JR., M. S. SIMPSON, A. R. WILSON, J. R. THOMPSON and H. E. SKIPPER, *Cancer Res.* **19**, 856 (1959).
24. J. A. JACQUEZ and F. GINSBERG, *Proc. Soc. exp. Biol. Med.* **105**, 478 (1960).
25. A. C. SARTORELLI and G. A. LEPAGE, *Cancer Res.* **18**, 1329 (1958).
26. L. L. BENNETT, JR., L. SIMPSON, J. GOLDEN and T. L. BARKER, *Cancer Res.* **23**, 1574 (1963).
27. H. P. SCHNEBLI, D. L. HILL and L. L. BENNETT, JR., *J. biol. Chem.* **242**, 1997 (1967).
28. J. F. HENDERSON, I. C. CALDWELL and A. R. P. PATERSON, *Cancer Res.* **27**, 1773 (1967).
29. J. F. HENDERSON, *Biochem. Pharmac.* **12**, 551 (1963).
30. D. L. HILL and L. L. BENNETT, JR., *Biochemistry, N.Y.* **8**, 122 (1969).
31. R. J. MCCOLLISTER, W. R. GILBERT, JR., D. M. ASHTON and J. B. WYNGAARDEN, *J. biol. Chem.* **239**, 1560 (1964).
32. R. P. MEICH, R. E. PARKS, JR., J. H. ANDERSON, JR. and A. C. SARTORELLI, *Biochem. Pharmac.* **16**, 2222 (1967).
33. R. P. MIECH, R. YORK, and R. E. PARKS, JR., *Molec. Pharmac.* **5**, 30 (1969).
34. M. T. HAKALA and C. A. NICHOL, *Biochim. biophys. Acta* **80**, 665 (1964).