Purine Ribonucleoside Kinase Activity and Resistance to Some Analogs of Adenosine

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SUMMARY

6-Methylthiopurine ribonucleoside, a cytotoxic nucleoside with antitumor activity, was phosphorylated by cell-free preparations from H.Ep. #2 cells in culture and from yeast. Studies of phosphorylation in the presence of other nucleosides indicated that adenosine kinase was the enzyme catalyzing this phosphorylation and also the phosphorylation of several other cytotoxic nucleosides derived from adenosine by substitution at the 2-position, substitution of other groups for the 6-amino group, or alteration of the ring structure. Cell lines resistant to 6-methylthiopurine ribonucleoside were selected from H.Ep. #2 cells and from a subline resistant to 6-mercaptopurine; a third line resistant to 2-fluoroadenosine was selected from a subline of H.Ep. #2 cells resistant to 2-fluoroadenine. Each of the three resistant cell lines was also resistant to a series of adenosine analogs that were toxic to the parent cell line. The resistant cells were devoid of detectable capacity to phosphorylate 6-methylthiopurine ribonucleoside and adenosine, and this loss persisted when the cells were cultured for many generations in the absence of inhibitor. The resistant cells did not differ from the cell lines from which they were derived in capacity to utilize purines. These results indicate that adenosine kinase is an enzyme essential for the activation of many cytotoxic nucleosides and that loss of this enzyme activity is a mechanism of resistance to these nucleosides.

INTRODUCTION

A number of ribonucucleosides are highly cytotoxic compounds with an interesting spectrum of biologic activity, including activity against certain experimental tumors. Among the more active of these compounds are 2-fluoroadenosine (1, 2), tubercidin (7-deazaadenosine) (3, 4), and the ribonucleoside of MeMP[2] (5). This last nucleoside, a simple derivative of MP-ribonucleoside, is of particular interest because of its activity against cells resistant to MP and MP-ribonucleoside (5). Since the active forms of the highly cytotoxic analogs of purines and nucleosides are the nucleotides (6), MeMP-ribonucleoside and other growth-inhibitory nucleosides must be activated intracellularly, presumably by one of the pathways shown below. Earlier studies (5) showed that (a) MeMP-ribonucleoside was rapidly converted to the nucleotide by H.Ep. #2 cells in culture and by cell-free extracts of these cells; (b) the corresponding base, MeMP, was not growth inhibitory and was not a substrate for purine nucleotide pyrophosphorylases; and (c) MeMP-ribonucleoside was not converted in significant amounts to the free base. These results suggested that MeMP-ribonucleoside was converted to the nucleotide by adenosine kinase, the only purine

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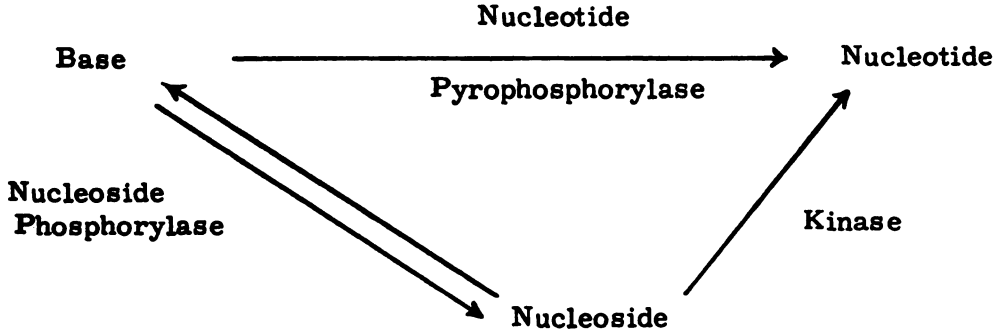
[2] The abbreviations used are: MP, 6-mercaptopurine; MeMP, 6-methylthiopurine.

Mol. Pharmacol. 2, 432–443 (1966)

nucleoside kinase known to occur in mammalian cells (7). The present paper is concerned with the nature of the kinase acting on MeMP-ribonucleoside, the changes in kinase activities in cell lines selected for resistance to MeMP-ribonucleoside or 2-fluoroadenosine, and the response of these resistant cell lines to other nucleoside analogs.



MATERIALS AND METHODS

Compounds. 6-Mercaptopurine-^{35}S ribonucleoside was prepared by exchange of ^{35}S with MP-ribonucleoside (5, 8) and was then alkylated to give ^{35}S-labeled ribonucleosides of MeMP, 6-ethylthiopurine, and 6-benzylthiopurine. Tubercidin was a gift from Dr. C. G. Smith of the Upjohn Company; samples of 7-deazainosine, the deamination product of tubercidin (9), gifts from Dr. C. G. Smith and from Dr. A. Bloch, Roswell Park Memorial Institute; and adenosine-1-*N*-oxide, previously shown to be cytotoxic to cultured cells (10), a gift from Dr. George B. Brown of the Sloan-Kettering Institute. 8-Azaadenosine was synthesized by Dr. Leon Goodman, Stanford Research Institute, and obtained through the Cancer Chemotherapy National Service Center, National Cancer Institute. References to the syntheses of these and other purines and nucleosides used in this study are given in Table 7. Phosphoenolpyruvate kinase and phosphoenolpyruvate were products of Sigma Chemical Company. Adenosine-8-^{14}C and inosine-8-^{14}C were obtained from Schwarz BioResearch, Inc., and adenine-8-^{14}C, hypoxanthine-8-^{14}C, and guanine-8-^{14}C from New England Nuclear Corporation.

Cell cultures. Cells of the human epidermoid carcinoma (H.Ep. #2) cell line of Moore *et al.* (11) were grown either in stationary or suspension culture as described elsewhere (12). By serial passage of these cells in the presence of gradually increasing concentrations of inhibitor, three resistant cell lines were selected for use in this study. For the first of these, resistance was developed to MeMP-ribonucleoside starting with the parent H.Ep. #2 line (hereafter designated H.Ep. #2/S to distinguish it from the resistant lines); the resistant line thus developed (designated H.Ep. #2/MeMPR) grew in the presence of 100 μg MeMP-ribonucleoside per milliliter, over 300-fold the concentration inhibiting growth of the parent line by 50%. For selection of the second resistant line, the starting cell line was one resistant to MP and devoid of IMP and GMP pyrophosphorylase activities (13). Culture of this line in the presence of MeMP-ribonucleoside led to a doubly resistant line, H.Ep. #2/MP/MeMPR, which was as resistant to MeMP-ribonucleoside as was the H.Ep. #2/MeMPR line. The third subline was selected for resistance to 2-fluoroadenosine starting with a cell line (H.Ep. #2/FA) resistant to 2-fluoroadenine and lacking AMP pyrophosphorylase. This doubly resistant line (H.Ep. #2/FA/FAR) grew well in a concentration of 2-fluoroadenosine of 70 μM or greater, whereas growth of the parent line was inhibited more than 50% by a concentration of 0.4 μM. The response of these cell lines to inhibitors was determined by cloning procedures described in detail in Table 7.

Protein assays. Protein was determined for all enzyme preparations by the method of Lowry *et al.* (14).

Assays for purine nucleotide pyrophosphorylases. Assays for IMP-, GMP-, and AMP-pyrophosphorylase activities were carried out with acetone powders prepared from cells grown either in stationary or suspension cultures. The procedures, given in Table 5, are essentially those used by Brockman and co-workers (13, 15).

Assays for nucleoside kinase. Cell-free preparations from both H.Ep. #2 cells and yeast were assayed for purine nucleoside kinase activity. Cells from the parent H.Ep. #2 cell line and the three resistant sublines, grown as described above, were collected by centrifugation and washed free of medium with 0.9% NaCl solution. The packed cells were suspended in 3 volumes of water and homogenized in a glass-Teflon homogenizer, and the homogenate was centrifuged at 25,000 *g* for 45 minutes. The supernatant, which contained 15–25 mg protein per milliliter, was used in most experiments without further purification. For the yeast enzyme, the starting material was dried "Brewer's Bottom Yeast" obtained from Sigma Chemical Company. This material was suspended in 3 volumes of water and allowed to stand in the cold (~0°) for 3–4 days (16). The suspension was then centrifuged at 25,000 *g* for 45 min; the opalescent supernatant was stored frozen (—20°) for 24 hr, thawed, and again centrifuged for 10 min at 25,000 *g*. The supernatant contained

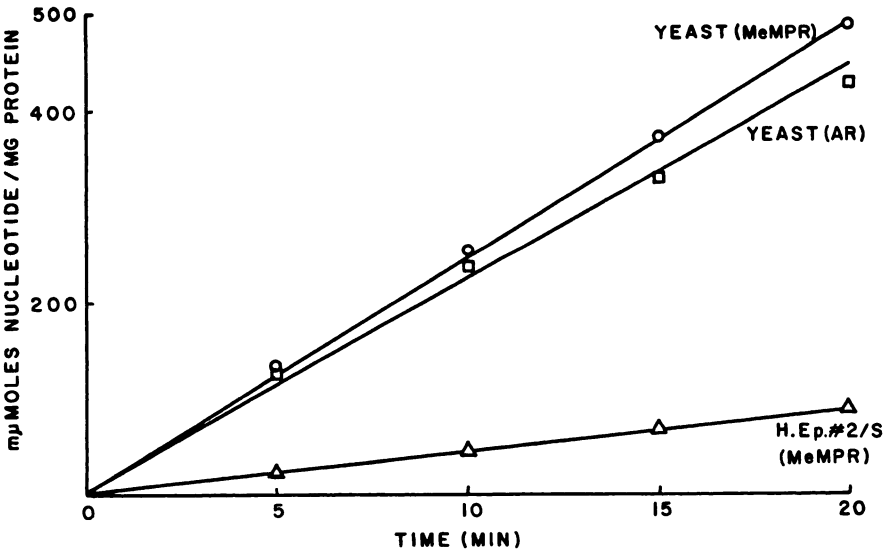


FIG. 1. *Phosphorylation of adenosine and 6-methylthiopurine ribonucleoside*

The incubation mixture consisted of the following in a final volume of 1.0 ml, pH 7.0: ATP, 2.5 μmoles; Mg^{++}, 0.25 μmole (H.Ep. #2 cells) or 2.5 μmoles (yeast); potassium phosphate buffer, pH 7.0 (50 μmoles); enzyme, 5.0 mg protein (H.Ep. #2 cells) or 1.0 mg (yeast), and the substrate, adenosine-8-^{14}C or MeMP-ribonucleoside-^{35}S, 1 μmole. Following incubation at 25°, the reaction was stopped by boiling for 1 min (H.Ep. #2 enzyme) or by the addition of 10 volumes of ethanol (yeast enzyme). Ethanol was used for deactivation of the yeast enzyme because it was found that heat deactivation gave nonreproducible results, presumably because of an increased rate of product formation during the period of heating. After denaturation of the protein, portions of the supernatant were subjected to chromatography in a butanol–propionic acid–water solvent prepared from equal volumes of 93.8% *n*-butanol and 44% aqueous propionic acid. The radioactive spots, located by radioautography, were cut out and assayed for ^{14}C or ^{35}S in a liquid scintillation spectrometer. With adenosine as substrate, the nucleotides formed represent the sum of AMP, ADP, and ATP; with MeMP-ribonucleoside as substrate, the monophosphate was the only nucleotide formed. Abbreviations: AR, adenosine; MeMPR, 6-methylthiopurine ribonucleoside.

15–30 mg protein per milliliter; it was also used without further purification. Neither the mammalian nor the yeast supernatant lost activity when stored frozen overnight. Dialysis caused a loss of most of the activity of both supernatants. Kinase assays were performed on these supernatants with adenosine-8-^{14}C, MeMP-^{35}S-ribonucleoside, 6-ethylthiopurine-^{35}S ribonucleoside, and 6-benzylthiopurine-^{35}S ribonucleoside as substrates, under the conditions described in Fig. 1.

Metabolism of purines and nucleosides by intact cells. For these studies, the labeled purine or nucleoside was added to cell cultures in logarithmic growth, the cells were collected at various times thereafter, and the soluble fractions and the polynucleotides were isolated and assayed as described elsewhere (17, 18). Further details are given in Tables 2 and 6.

RESULTS

Phosphorylation of Nucleosides

Methylthiopurine ribonucleoside was a substrate for a nucleoside kinase in preparations obtained from both H.Ep. #2 cells and yeast, that from yeast having the higher activity (Fig. 1). For the preparations from yeast, the rates of phosphorylation of MeMP-ribonucleoside and of adenosine were about the same. A similar

TABLE 1

Some factors influencing the nucleoside kinase reaction

See Fig. 1 for procedures for the assay and the components of the complete incubation mixture.

	mμMoles nucleotide formed/min/mg protein from:		
	MeMP-ribonucleoside		Adenosine
Sample	H.Ep. #2	Yeast	Yeast
Complete system	4.2	28.5	27.5
Enzyme boiled 2 min before reaction	<0.1	<0.1	<0.1
Mg^{++} omitted	3.6	4.7	11.7
ATP omitted	1.0	1.5	1.0
1/5 ATP + ATP regenerating system (0.01 mg PEP kinase;[a] 0.2 mg PEP)	3.2	26.6	25.8

[a] 1 mg converts 250 μmoles PEP to pyruvate per minute at pH 7.6 and 37°.

TABLE 2

Conversion of 6-alkylthiopurine ribonucleosides to nucleotides by H.Ep. #2 cells and cell-free extracts

The studies of phosphorylation in the cell-free extract were carried out by the procedures described in Fig. 1. For studies of phosphorylation in intact cells, the labeled nucleoside was added to suspension cultures (about 10^6 cells/ml) at a concentration of 32 mμmoles/ml. Four hours later the cells were collected by centrifugation, washed free of medium with 0.9% NaCl solution, and extracted with hot 80% aqueous ethanol. The water-soluble portion of this extract was subjected to paper chromatography in butanol-propionic acid solvent (equal volumes of 93.8% aqueous *n*-butanol and 44% aqueous propionic acid); the radioactive spots were cut out and assayed for ^{35}S in a liquid scintillation spectrometer. 6-Methylthiopurine ribonucleotide was identified by (a) its migration like an authentic, synthetic sample of 6-methylthiopurine ribonucleotide upon paper chromatography in four additional solvents varying widely in pH and upon paper electrophoresis in formate buffer, pH 3.5, and borate buffer, pH 9.2; (b) its conversion by crude snake venom (*Crotalus atrox*) to a compound migrating like MeMP-ribonucleoside; and (c) its conversion by acid hydrolysis to a compound migrating like MeMP. The 6-ethylthiopurine and 6-benzylthiopurine ribonucleotides were characterized by their conversion to the ribonucleosides upon treatment with snake venom.

Precursor and specific activity	Intact cells (cpm/10^6 cells present as nucleotide)	Cell-free extract (mμmoles nucleotide formed by 1 mg enzyme protein/min)
6-Methylthiopurine-^{35}S ribonucleoside (2.77 mC/mmole)	11,848	3.7
6-Ethylthiopurine-^{35}S ribonucleoside (0.96 mC/mmole)	1,816	0.4
6-Benzylthiopurine-^{35}S ribonucleoside (1.28 mC/mmole)	591	<0.2

Mol. Pharmacol. 2, 432–443 (1966)

TABLE 3

Effects of various nucleosides on the phosphorylation of adenosine and 6-methylthiopurine ribonucleoside by enzyme preparations from yeast and from H.Ep. #2 cells

Nucleotide formation was measured at the end of 10 min incubation under the same conditions described in Fig. 1 except for the addition of the competitive substrate.

Enzyme	Substrate and concentration	Concentration of competitive substrate (μmoles/ml)	Conversion of substrate to nucleotide in presence of competitive substrates[a]									
			MeMP-ribonucleoside	Adenosine	Inosine	Guanosine	2-Fluoroadenosine	Purine ribonucleoside	6-Hydrazinopurine ribonucleoside	8-Azaguanosine	Tubercidin	7-Deazainosine
Yeast	Adenosine-8-^{14}C, 1 μmole/ml	0.1	96		95	91	102	100	110	97	96	104
		1	91		98	102	102	103	89	100	86	102
		10	53		104	96	81	85	46	91	59	99
	MeMP-^{35}S-ribonucleoside, 1 μmole/ml	0.1		55	101	100	109	78	82	107	96	108
		1		1	97	110	90	41	38	107	66	102
		10		<1	106	101	50	6	5	100	21	91
H.Ep. #2/S	MeMP-^{35}S-ribonucleoside, 1 μmole/ml	0.1		85	102	99	98	99	84		67	
		1		9	97	95	96	77	53		15	
		10		<1	81	87	54	23	8		1	93

[a] The results are presented as percentages of the amounts of nucleotides formed from the substrate in the absence of a competitive substrate.

quantitative comparison could not be made in H.Ep. #2 extracts because of the high activity of adenosine deaminase: although some nucleotides were formed from adenosine, the predominant products were inosine and hypoxanthine. No adenosine deaminase activity was found in the yeast extract. As expected, omission of ATP or Mg^{++} decreased the production of nucleotides (Table 1). Lowering the concentration of the ATP and addition of a regenerating system did not change the rate of nucleotide formation. The pH optima determined in universal buffer (19) were: MeMP-ribonucleoside, 7.5 (yeast), 6.5–7.0 (H.Ep. #2 cells); adenosine, 7.0 (yeast). Kornberg and Pricer (20) assayed their yeast kinase at pH 6.0; Caputto (21) found a pH optimum of 6.9–7.0 for his yeast kinase. Our observed optimum in H.Ep. #2 cell extracts is higher than those found in other types of mammalian cells: an optimum of 4.9–5.0 was observed for the phosphorylation of adenosine by liver and kidney extracts (21) and an optimum of 5.4 for the phosphorylation of MeMP-ribonucleoside by an extract of Ehrlich ascites cells (22). These discrepancies appear to be a result of differences in buffers, since assay of the H.Ep. #2 enzyme in succinate buffer gave a pH optimum of 5.5 for the phosphorylation of MeMP-ribonucleoside.

In intact cells 6-methyl-, 6-ethyl-, and 6-benzylthiopurine ribonucleosides were all phosphorylated, but the ethyl and benzyl derivatives to a lesser extent than the methyl derivative (Table 2). The relative amounts of nucleotides formed from these three compounds in both intact cells and in cell-free systems are the same as their relative toxicities to H.Ep. #2 cells (5) (see also Table 7).

Phosphorylation of Substrates in the Presence of Other Nucleosides

To obtain information on the specificity of the nucleoside kinase, a series of nucleosides was examined for capacity to interfere with the phosphorylation of adenosine and MeMP-ribonucleoside in yeast extracts and with MeMP-ribonucleoside in H.Ep. #2 extracts (Table 3). Only MeMP-ribonucleoside, 6-hydrazinopurine ribonucleoside, and tubercidin markedly decreased the phosphorylation of adenosine, and then only when present in concentrations 10-fold that of adenosine. When MeMP-ribonucleoside was the substrate, adenosine almost completely prevented phosphorylation when present at the same concentration as that of MeMP-ribonucleoside. Other compounds decreasing the phosphorylation of MeMP-ribonucleoside were purine ribonucleoside, 6-hydrazinopurine ribonucleoside, tubercidin, and 2-fluoroadenosine; the same compounds that interfered with phosphorylation by the yeast kinase also interfered with phosphorylation by the H.Ep. #2 kinase. Inosine, guanosine, 8-azaguanosine, and 7-deazainosine did not interfere with the phosphorylation of either substrate. Caldwell *et al.* (22) have also concluded that adenosine and MeMP-ribonucleoside are phosphorylated by the same kinase in Ehrlich ascites cells.

Nucleoside Kinase Activities of Parent and Resistant Cell Lines

None of the cell lines had detectable capacity to phosphorylate inosine or guanosine. Whereas the parent cell line had kinase activity for both adenosine and MeMP-ribonucleoside, all three of the resistant lines had lost capacity to phosphorylate either of these nucleosides in detectable amounts (Table 4). The ratio of capacities of sensitive to capacities of resistant cells is >50 for adenosine, and >400 for MeMP-ribonucleoside. The stability of the resistance and of the loss of kinase is shown by the failure of kinase activity to reappear when the cells were cultured for many generations in the absence of drug.

Nucleotide Pyrophosphorylase Activities (Table 5)

The parent and H.Ep. #2/MeMPR cell lines did not differ significantly in the activities of adenylic, inosinic, and guanylic pyrophosphorylases. The H.Ep. #2/MP/MeMPR line showed a specific loss of inosinic and guanylic pyrophosphorylase activities and the H.Ep. #2/FA/FAR line a

TABLE 4

Nucleoside kinase activity of H.Ep. #2 cells and various sublines

Assays for kinase activities were carried out under the conditions given in Fig. 1. The designation "/N" indicates that the cells used in this assay had been carried in the absence of inhibitor for 10 generations or longer. Inosine-8-^{14}C and guanosine-8-^{14}C were also studied as substrates; for all cell lines there was no detectable formation (i.e., values <0.6) of nucleotides.

Cell line	Nucleotide formation (mμmoles/hr/mg protein)	
	From adenosine-8-^{14}C	From 6-methylthiopurine-^{35}S ribonucleoside
H.Ep. #2/S	30–60[a]	240–300
H.Ep. #2/MeMPR	<0.6	<0.6
H.Ep. #2/MeMPR/N	<0.6	<0.6
H.Ep. #2/MP/MeMPR	<0.6	<0.6
H.Ep. #2/MP/MeMPR/N	<0.6	<0.6
H.Ep. #2/FA/FAR	<0.6	<0.6
H.Ep. #2/FA/FAR/N	<0.6	<0.6

[a] Although most of the adenosine was degraded to inosine and hypoxanthine, significant amounts of adenine nucleotides were found.

TABLE 5

Purine nucleotide pyrophosphorylase activities of H.Ep. #2 cells and sublines resistant to 6-methylthiopurine ribonucleoside or 2-fluoroadenosine

The reaction mixture contained 5-phosphoribosyl 1-pyrophosphate (1 μmole); Tris (50 μmoles); ^{14}C-labeled adenine, guanine, or hypoxanthine (0.25 μmole); the cell-free supernatant (about 200 μg protein), and H_2O in a final volume of 0.5 ml; pH 7.6. The reaction was stopped by the addition of EDTA and immersion in boiling water. The radioactive nucleotides were isolated by paper chromatography (70% aqueous isopropanol in an ammonia atmosphere), and the radioactive spots, located by radioautography, were cut out and assayed for ^{14}C in a Packard Tri-Carb liquid scintillation counter.

Cell line	mμMoles nucleotides formed from corresponding free base per mg protein per 20 min		
	AMP	GMP	IMP
H.Ep. #2/S	131	377	158
H.Ep. #2/MeMPR	140	340	160
H.Ep. #2/MP/MeMPR	112	15	4
H.Ep. #2/FA/FAR	2	408	200

loss of adenylic pyrophosphorylase activity. These losses in enzyme activity are the same as those in the cell lines (H.Ep. #2/MP and H.Ep. #2/FA) from which the two doubly resistant lines were derived; these results therefore show that no change in pyrophosphorylase activities occurred during the process of selection for resistance to MeMP-ribonucleoside and 2-fluoroadenosine.

Incorporation of Purines and Nucleosides into Polynucleotides (Table 6).

Of the four cell lines assayed, only the H.Ep. #2/FA/FAR line failed to incorporate adenine to a significant extent, and only the H.Ep. #2/MP/MeMPR line failed to incorporate hypoxanthine. These results are those to be expected from the observed activities for nucleotide pyrophosphorylases. Adenosine was incorporated well by the H.Ep. #2/S and H.Ep. #2/FA/FAR cell lines and moderately well by the H.Ep. #2/MeMPR line; the incorporation by H.Ep. #2/MP/MeMPR cells was much lower, although it was still significantly greater than the incorporation of hypoxanthine or inosine. This finding suggests (a) that some adenosine is phosphorylated directly in intact cells, but that a major route of conversion of adenosine to nucleotides is adenosine → inosine → hypoxanthine → IMP → AMP + GMP, a route not operative in the H.Ep. #2/MP/MeMPR cell line because of its deficiency of IMP pyrophosphorylase, and, (b) that the conversion of adenosine to adenine occurs, if at all, to a very small extent. Analysis of the soluble fractions from these cells by two-dimensional chromatography and radioautography gave results consistent with those on incorporation into polynucleotides: nucleotides were not formed in significant amounts from adenine in H.Ep.

TABLE 6

Incorporation of purines and nucleosides into polynucleotides of H.Ep. #2 cell lines

To suspension cultures (10^6 cells/ml) in logarithmic growth were added the ^{14}C-labeled precursors to give a final concentration of 39 mμmoles/ml (11 mμC/ml) in the cell culture medium. Four hours thereafter the cells were harvested by centrifugation, washed free of medium with 0.9% NaCl solution, and extracted with boiling 80% aqueous ethanol. Crude sodium nucleates, isolated from the residue with hot 10% NaCl solution, were assayed for polynucleotide content by ultraviolet spectrophotometric analysis and assayed for ^{14}C content in a liquid scintillation spectrometer. The values given are for the results of a single experiment, which were confirmed in a second experiment in which the concentrations of precursors were different.

Cell line	Specific activities of polynucleotides (mμC/mg)			
	Hypoxanthine-8-^{14}C	Inosine-8-^{14}C	Adenine-8-^{14}C	Adenosine-8-^{14}C
H.Ep. #2/S	12.6	13.6	21.4	24.8
H.Ep. #2/MeMPR	10.9	8.5	5.7	7.6
H.Ep. #2/MP/MeMPR	<0.1	0.5	23.5	3.0
H.Ep. #2/FA/FAR	24.0	19.8	0.2	42.9

#2/FA/FAR cells or from hypoxanthine or inosine in the H.Ep. #2/MP/MeMPR cells. Since these results only confirm those on polynucleotide formation, they are not presented here.

Response of Cell Lines to Inhibitors (Table 7)

The H.Ep. #2/MeMPR and the H.Ep. #2/FA/FAR lines were assayed for response to a series of purines, purine analogs, and their nucleosides. The cell line selected for resistance to MeMP-ribonucleoside was resistant to the other adenosine analogs with the exception of 2-fluoroadenosine, and that selected for resistance to 2-fluoroadenosine was resistant to MeMP-ribonucleoside and all the adenosine analogs—results consistent with the finding that both resistant cell lines had lost kinase activity for both adenosine and MeMP-ribonucleoside. The degree of resistance to 8-azaadenosine was much less than the degree of resistance to the other adenosine analogs. Both resistant cell lines were resistant to 7-deazainosine. The pattern of response to the free bases and to MP-ribonucleoside was that to be expected from the known changes in pyrophosphorylase activities: H.Ep. #2/MeMPR cells, which contained normal amounts of all the nucleotide pyrophosphorylases, showed little or no resistance to 2-fluoroadenine, 4-aminopyrazolo[3, 4-d]pyrimidine, and MP, whereas H. Ep. #2/FA/FAR cells were resistant to the adenine analogs but sensitive to MP. The resistant cells were not cross resistant to 5-fluorodeoxyuridine.

DISCUSSION

The loss of nucleoside kinase activity in all three cell lines selected for resistance to MeMP-ribonucleoside or 2-fluoroadenosine and the consequent cross-resistance to other nucleosides indicate that a nucleoside kinase is essential for the activation of these nucleoside analogs. It would be expected that the kinase involved is adenosine kinase, which is the only purine nucleoside kinase known to occur widely (7, 23).[3] The results of Table 4, which show that inosine, guanosine, and their analogs did not de-

[3] Kinases for inosine and guanosine have been reported in salmon milt (24) but have not been found in mammalian cells (25). However, certain tumor cells apparently have the capacity to phosphorylate both the α- and β-anomers of 2'-deoxy-6-thioguanosine (26), and some 6-thioguanosine was converted to the nucleotide by cells that lacked the necessary pyrophosphorylase activity for conversion of the free base to nucleotide (27). Brewers' yeast has a kinase (which may be adenosine kinase) that catalyzes the phosphorylation of the ribonucleoside of 4-aminoimidazole-5-carboxamide (28, 29).

TABLE 7

Effects of a series of purines and nucleosides on growth of H.Ep. #2 cells and sublines resistant to 6-methylthiopurine ribonucleoside or 2-fluoroadenosine

Approximately 100 cells were placed in 4-ounce prescription bottles containing for control cultures 10 ml of SRI-14 medium (12) and for treated cultures 10 ml of medium in which the inhibitor was present at the desired concentrations. After the cultures had been incubated at 37° without disturbance for 7–10 days, the medium was decanted, and the cells adhering to the glass were washed with phosphate-buffered 0.85% NaCl solution (pH 7.0), fixed with Bouin's fixative, and stained with Giemsa stain. The number of macroscopic colonies that had formed were then counted. In control cultures, cloning efficiency was usually in the range of 40–60%.

Compound (and reference to synthesis)	Inhibitory concentrations,[a] H.Ep. #2/S cells (μmoles/l)	Ratio of inhibitory concentrations[b]	
		H.Ep. #2/MeMPR / H.Ep. #2/S	H.Ep. #2/FA/FAR / H.Ep. #2/S
2-Fluoroadenine (39)	0.03	1	>2000
2-Fluoroadenosine (1)	0.02	1–2	>2000
4-Aminopyrazolo[3,4-*d*]pyrimidine (40)	3	2	>25
4-Aminopyrazolo[3,4-*d*]pyrimidine ribonucleoside (41)	0.2	>300	>330
Tubercidin (9, 42)	0.002	>1400	>2000
8-Azaadenosine (43)	0.8	2–3	2–3
7-Deazainosine (44)	2	>20	>10
6-Hydrazinopurine ribonucleoside (45)	2	>80	>80
Purine ribonucleoside (46)	0.08	>500	>500
6-Chloropurine ribonucleoside (46)	3	>20	>20
2-Chloroadenosine (47)	7	>10	>10
Adenosine-1-*N*-oxide (48)	3	>50	>50
6-Methylthiopurine ribonucleoside (49)	1	>300	>300
6-Ethylthiopurine ribonucleoside (50)	2	>100	>100
6-Benzylthiopurine ribonucleoside (50)	8	>7	>7
6-Mercaptopurine	0.6	0.5–1	0.5–1
6-Mercaptopurine ribonucleoside (51)	0.7	1	1
5-Fluorodeoxyuridine	2	0.4–0.6	0.4

[a] Concentrations inhibiting colony formation by more than 50%.

[b] The ">" values indicate no significant inhibition of the resistant cell line at the highest concentration of inhibitor assayed.

crease the phosphorylation of adenosine, whereas MeMP-ribonucleoside and several of the nucleosides that are structural analogs of adenosine did, are generally consistent with adenosine kinase being the enzyme responsible for the phosphorylation. In view of the fact that 7-deazainosine did not interfere with the phosphorylation of adenosine or MeMP-ribonucleoside, it is surprising that it did not inhibit the kinase-deficient cell lines: the degree of resistance to deazainosine was comparable to that to many of the adenosine analogs (Table 7). These results suggest the existence of a kinase other than adenosine kinase; such a kinase, if it exists, must be closely linked with adenosine kinase because the cell lines resistant to adenosine analogs, presumably as a result of loss of activity of adenosine kinase, were also highly resistant to 7-deazainosine. The fact that the resistant cells were still sensitive to 5-fluorodeoxyuridine, which is activated by thymidine kinase (30), indicates that all nucleoside kinase activity has not been lost.

If one assumes that failure of a nucleoside to inhibit the kinase-deficient cell lines is evidence that the nucleoside is a substrate for the lost kinase(s), then it is apparent that a variety of structural changes can be

made in adenosine with retention of capacity to act as a substrate for the kinase. Thus, an amino group at the 6-position is not essential, as may be seen from the results with the ribonucleosides of MeMP, 6-ethylthiopurine, 6-benzylthiopurine, 6-chloropurine, and purine. Substitution of fluorine or chlorine at the 2-position, alterations in the purine ring (cf. 4-aminopyrazolo[3,4-*d*]pyrimidine ribonucleoside, tubercidin, and 8-azaadenosine) or oxidation of the 1-*N*-atom also resulted in nucleosides that apparently are substrates for the kinase. The only reported study of substrate specificity for adenosine kinase is that of Kornberg and Pricer (20), who found that, with yeast kinase, of a series of nucleosides only adenosine and 2,6-diaminopurine ribonucleoside were substrates. There are two other recent reports relating to the phosphorylation of some of the nucleosides of Table 7. Tubercidin has been found to be phosphorylated and incorporated into polynucleotides of mouse fibroblasts (31), and 2-fluoroadenosine was converted in Ehrlich cells to mono-, di-, and triphosphates (32).

A nucleoside conceivably could be converted to the nucleotide by the sequential action of a nucleoside phosphorylase and a nucleotide pyrophosphorylase, a route that is apparently operative in the conversion of MP-ribonucleoside to the nucleotide (33) and of 6-thioguanosine to 6-thioguanylic acid (27). The existence of such a pathway for the activation of 2-fluoroadenosine is suggested by the fact that H.Ep. #2/MEMPR cells, which had lost kinase activity but not AMP pyrophosphorylase activity, were not cross-resistant to 2-fluoroadenosine. [2-Fluoroadenosine has in fact been shown to be a good substrate for the adenosine phosphorylase of *Streptococcus faecalis* (34).] That such a pathway does not exist to a significant extent for the other toxic adenosine analogs is illustrated by the consistent resistance of H.Ep. #2/MeMPR cells to these analogs. With regard to the cleavage of fluoroadenosine to fluoroadenine, it is noteworthy that lines of *Escherichia coli* selected for resistance to 2-fluoroadenine or 2-fluoroadenosine were each resistant to both compounds, and that both lines had decreased AMP pyrophosphorylase activity (35).

A striking feature of the data with 8-azaadenosine (Table 7) is the relatively low resistance of the kinase-deficient cell lines to this analog. These results suggest that 8-azaadenosine may be inhibitory as such, that is, without conversion to the nucleotide, or that there is an alternative route for its activation. Since 8-azahypoxanthine has about the same cytotoxicity as 8-azaadenosine,[4] a conceivable route is that represented by the sequence 8-azaadenosine → 8-azainosine → 8-azahypoxanthine → 8-aza-IMP.

Although the association of loss of purine nucleoside kinase activity with resistance to cytotoxic nucleosides is not positive proof of a causal relationship, the consistency of the results suggest that such is the case. In the area of pyrimidine metabolism a similar mechanism of resistance has been found: cell lines selected for resistance to 5-fluorodeoxyuridine (36) or to 5-bromodeoxyuridine (37) had lost thymidine kinase activity and lines resistant to 1-β-D-arabinofuranosylcytosine had lost deoxycytidine kinase activity (38). A mechanism that is qualitatively the same, namely loss of the enzyme responsible for formation of the nucleotide, has also been shown to explain resistance to analogs of purines, which in a number of biologic systems was associated with the loss of a purine nucleotide pyrophosphorylase (6). It is noteworthy that as far as is known, neither purine nucleoside kinase(s) nor purine nucleotide pyrophosphorylases are essential for growth of mammalian cells, but apparently represent only salvage pathways that enable the cell to utilize preformed purines if they are available. The nonessentiality for growth of enzymes that are essential for activation of the inhibitors makes it possible for cells to become highly resistant to analogs of purines and nucleosides without impairment of their capacity for normal growth.

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[4] G. J. Dixon and J. A. Montgomery, unpublished observations, 1966.

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REFERENCES

1. J. A. Montgomery and K. Hewson, *J. Am. Chem. Soc.* **79**, 4559 (1957).
2. F. R. White, *Cancer Chemotherapy Rept.* **11**, 236 (1961).
3. S. P. Owen and C. G. Smith, *Cancer Chemotherapy Rept.* **36**, 19 (1964).
4. L. R. Duvall, *Cancer Chemotherapy Rept.* **30**, 61 (1963).
5. L. L. Bennett, Jr., R. W. Brockman, H. P. Schnebli, S. Chumley, G. J. Dixon, F. M. Schabel, Jr., E. A. Dulmadge, H. E. Skipper, J. A. Montgomery, and H. J. Thomas, *Nature* **205**, 1276 (1965).
6. R. W. Brockman, *Advan. Cancer Res.* **7**, 129 (1963).
7. R. Caputto, *in* "The Enzymes" (P. D. Boyer, H. Lardy and K. Myrbäck, eds.), 2nd ed., Vol. 6, p. 133. Academic Press, New York, 1962.
8. J. Moravek and Z. Nejedly, *Chem. Ind.* p. 530 (1960).
9. S. Suzuki and S. Murumo, *J. Antibiotics (Tokyo) Ser. A* **14**, 34 (1961).
10. G. B. Brown, D. A. Clarke, J. J. Biesele, L. Kaplan and M. A. Stevens, *J. Biol. Chem.* **233**, 1509 (1958).
11. A. E. Moore, L. Sabachewsky and H. W. Toolan, *Cancer Res.* **15**, 598 (1955).
12. G. G. Kelley, M. H. Vail, D. J. Adamson and E. A. Palmer, *Am. J. Hyg.* **73**, 231 (1961).
13. R. W. Brockman, G. G. Kelley, P. Stutts and V. Copeland, *Nature* **191**, 469 (1961).
14. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.* **193**, 265 (1951).
15. R. W. Brockman, C. S. Debavadi, P. Stutts and D. J. Hutchison, *J. Biol. Chem.* **236**, 1471 (1961).
16. H. Z. Sable, *Proc. Soc. Exptl. Biol. Med.* **75**, 215 (1950).
17. E. P. Anderson and R. W. Brockman, *Biochem. Pharmacol.* **12**, 1335 (1963).
18. A. J. Tomisek, H. J. Kelly and H. E. Skipper, *Arch. Biochem.* **64**, 437 (1956).
19. H. T. S. Britton and G. Welford, *J. Chem. Soc.* p. 1848 (1937).
20. A. Kornberg and W. E. Pricer, *J. Biol. Chem.* **193**, 481 (1951).
21. R. Caputto, *J. Biol. Chem.* **189**, 801 (1951).
22. I. C. Caldwell, J. F. Henderson and A. R. P. Paterson, *Can J. Biochem.* **44**, 229 (1966).
23. H. E. Franz, M. Franz and K. Decker, *Z. Physiol. Chem.* **327**, 243 (1962).
24. H. L. A. Tarr, *Can. J. Biochem.* **42**, 1535 (1964).
25. M. F. Utter, *in* "The Enzymes" (P. D. Boyer, H. Lardy and K. Myrbäck, eds.), 2nd ed., Vol. 2, p. 75. Academic Press, New York, 1960.
26. G. A. LePage, I. G. Junga and B. Bowman, *Cancer Res.* **24**, 835 (1964).
27. G. A. LePage and I. G. Junga, *Cancer Res.* **23**, 739 (1963).
28. G. R. Greenberg, *J. Biol. Chem.* **219**, 423 (1956).
29. H. T. Huang, *Biochemistry* **4**, 58 (1965).
30. R. Okazaki and A. Kornberg, *J. Biol. Chem.* **239**, 269 (1964).
31. G. Acs, E. Reich and M. Mori, *Proc. Natl. Acad. Sci. U. S.* **52**, 493 (1964).
32. H. T. Shigeura, G. E. Boxer, S. D. Sampson and M. L. Meloni, *Arch. Biochem. Biophys.* **111**, 713 (1965).
33. A. R. P. Paterson and A. Sutherland, *Can. J. Biochem.* **42**, 1415 (1964).
34. A. Bloch and C. A. Nichol, *Antimicrobial Agents and Chemotherapy—1964*, 530 (1965).
35. R. F. Pittillo, C. Moncrief, R. W. Brockman and P. Chambers, *Antimicrobial Agents and Chemotherapy—1964*, 474 (1965).
36. N. R. Morris and G. A. Fischer, *Biochim. Biophys. Acta* **68**, 84 (1963).
37. S. Kit, D. R. Dubbs, L. J. Piekarski and T. C. Hsu, *Exptl. Cell Res.* **31**, 297 (1963).
38. M. Y. Chu and G. A. Fischer, *Biochem. Pharmacol.* **14**, 333 (1965).
39. J. A. Montgomery and K. Hewson, *J. Am. Chem. Soc.* **82**, 463 (1960).
40. R. K. Robins, *J. Am. Chem. Soc.* **78**, 784 (1956).
41. J. A. Montgomery, S. J. Clayton and W. E. Fitzgibbon, Jr., *J. Heterocyclic Chem.* **1**, 215 (1964).
42. K. Anzai, G. Nakamura and S. Suzuki, *J. Antibiotics (Tokyo) Ser. A* **10**, 201 (1957).
43. J. Davoll, *J. Chem. Soc.* p. 1593 (1958).
44. Y. Mizuno, M. Ikehara, K. A. Watanabe and S. Suzaki, *J. Org. Chem.* **28**, 3331 (1963).
45. J. A. Johnson, H. J. Thomas and H. J. Schaeffer, *J. Am. Chem. Soc.* **80**, 699 (1958).

46. G. B. Brown and V. S. Weliky, *J. Biol. Chem.* **204**, 1019 (1953).
47. J. A. Montgomery and K. Hewson, *J. Heterocyclic Chem.* **1**, 213 (1964).
48. M. A. Stevens, D. I. Magrath, H. W. Smith and G. B. Brown, *J. Am. Chem. Soc.* **80**, 2755 (1958).
49. A. Hampton, J. J. Biesele, A. E. Moore and G. B. Brown, *J. Am. Chem. Soc.* **78**, 5695 (1956).
50. J. A. Montgomery, T. P. Johnson, A. Gallagher, C. R. Stringfellow and F. M. Schabel, Jr., *J. Med. Pharm. Chem.* **3**, 265 (1961).
51. J. A. Johnson, Jr., and H. J. Thomas, *J. Am. Chem. Soc.* **78**, 3863 (1956).