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Metabolism and Metabolic Effects of 8-Aza-6-Thioinosine and its Rearrangement Product, N-β-D-Ribofuranosyl-[1,2,3]thiadiazolo[5,4-*d*]-pyrimidin-7-amine

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SUMMARY

BENNETT, L. LEE, JR., LUCY M. ROSE, PAULA W. ALLAN, DONALD SMITHERS, DORIS J. ADAMSON, ROBERT D. ELLIOTT AND JOHN A. MONTGOMERY. Metabolism and metabolic effects of 8-Aza-6-thioinosine and its rearrangement product, N-β-D-Ribofuranosyl[1,2,3]thiadiazolo[5,4-*d*]pyrimidin-7-amine. *Mol. Pharmacol.* **16**: 981-996, (1979).

8-Aza-6-thioinosine (8-aza-MPR), synthesized as an analogue of 6-thioinosine (MPR) and expected to have improved metabolic properties, was found, as predicted, to be a substrate for adenosine kinase (EC 2.7.1.20) but not for purine nucleoside phosphorylase (EC 2.4.2.1). In contrast MPR is known to be converted to its nucleotide via the free base. Both 8-aza-MPR and its rearrangement product, N-β-D-ribofuranosyl[1,2,3]thiadiazolo[5,4-*d*]pyrimidin-7-amine (TPR), were cytotoxic; the 50% inhibitory concentrations for H.Ep. #2 cells were 1.8 μM and 0.14 μM, respectively. Like 8-aza-MPR, TPR was a substrate for adenosine kinase but not for purine nucleoside phosphorylase. H.Ep. #2 cells grown in the presence of 8-aza-MPR contained three new nucleotides: the 5'-monophosphate of 8-aza-MPR and the 5'-monophosphates of the α- and β-anomers of TPR. Cells grown in the presence of TPR contained only the latter two metabolites. These nucleotides result from the phosphorylation of 8-aza-MPR and TPR, the rearrangement of 8-aza-MPR and its phosphate to TPR and TPR phosphate, and the anomerization of TPR phosphate. No di- or tri-phosphates were detected with either precursor. The total amount of nucleotides derived from TPR was of the order of 2.5 μmoles/10^9 cells, an amount 10–15 fold greater than the amount of nucleotides derived from 8-aza-MPR. There was no detectable incorporation of [^{35}S] from [^{35}S]-labeled-8-aza-MPR or TPR into polynucleotides. There was little or no desulfuration of 8-aza-MPR as determined by the absence of 8-aza-GTP, the principal soluble metabolite of 8-azainosine. Both 8-aza-MPR and TPR inhibited the synthesis of RNA and DNA but not of protein. Both reduced selectively the pools of guanine nucleotides in H.Ep. #2 cells. In addition 8-aza-MPR, like 8-azainosine, caused an accumulation of orotidine. The cytotoxicity of TPR to H.Ep. #2 cells was prevented by hypoxanthine; the cytotoxicity of 8-aza-MPR was prevented by a combination of hypoxanthine and uridine but not by either agent alone. These results indicate that a primary blockade produced by TPR was at some step of the synthesis of

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purine nucleotides, whereas 8-aza-MPR inhibited synthesis of both purine nucleotides (as a result of its conversion to TPR phosphates) and pyrimidine nucleotides (as a result of its conversion to 8-aza-MPR phosphate). Although both 8-aza-MPR and TPR are new biologically active nucleoside analogues, TPR is of particular interest because of its unusual structure and the evidence that it functions as an adenosine analogue despite the fact that its ribosyl moiety is attached not to a heterocyclic ring but to a primary amino group.

INTRODUCTION

6-Mercaptopurine (MP)[1], like most analogues of purines and nucleosides, inhibits cell proliferation only after its conversion to the nucleotide, a reaction catalyzed by hypoxanthine(guanine)phosphoribosyltransferase (H(G)PRT) (1). Many analogues and derivatives of MP have been synthesized in a search for agents that might have improved therapeutic activity, particularly agents that might circumvent resistance to MP arising from loss of activity of H(G)PRT. Among such compounds was MP-ribonucleoside, which was expected to be phosphorylated directly and therefore to be effective against cells that had lost capacity to convert the free base to the nucleotide (2). Cells resistant to MP as a result of loss of H(G)PRT activity, however, were equally resistant to MPR (1), a fact leading to the conclusion that MPR is converted to the nucleotide only via formation of MP. The failure of MPR to be phosphorylated directly could be due to 1) a low cellular activity of the required nucleoside kinase, 2) a high cellular activity of purine nucleoside phosphorylase (PNP), or 3) a combination of these factors.

A lead as to how MPR might be modified structurally so that it would be phosphorylated directly was derived from the observation that whereas inosine was not a substrate for adenosine kinase (AK), 8-azainosine was (3). This finding indicated that 8-aza-MPR should also be a substrate for AK; we have already reported that this nucleoside was in fact a good substrate for AK from H.Ep#2 cells (3). In the course of our work it was noted that this compound underwent a facile rearrangement to a thiadiazolopyrimidine, (Figs. 1, 10) (4), a type of rearrangement that has been studied earlier by Albert, Brown and their co-workers (5–7). The rearrangement product (TPR) itself showed biological activity and was in fact more toxic than 8-aza-MPR. We report here a study of the metabolism and metabolic effects of these related nucleosides. Preliminary reports of some of these results have been presented (8, 9).

[1] The abbreviations used are: MP, 6-mercaptopurine; MPR, 6-thioinosine; 8-aza-MP, 8-aza-6-mercaptopurine; 8-aza-MPR, 8-aza-6-thioinosine; MeMPR, 6-methylthioinosine; TP, [1,2,3]thiadiazolo[5,4-*d*]pyrimidin-7-amine; TPR, N-β-D-ribofuranosyl[1,2,3]thiadiazolo[5,4-*d*]pyrimidin-7-amine; 8-azaG, 8-azaguanine; AK, adenosine kinase (EC 2.7.1.20); H(G)PRT, hypoxanthine(guanine)phosphoribosyltransferase (EC 2.4.2.8); PNP, purine nucleoside phosphorylase (EC 2.4.2.1); HPLC, high pressure liquid chromatography; TLC, thin layer chromatography.

MATERIALS AND METHODS

8-Aza-MPR, TPR, [^{35}S]-8-Aza-MPR and [^{35}S]TPR. The syntheses of 8-aza-MPR (4, 10) and of TPR (4) have been reported. In the course of devising a satisfactory procedure for [^{35}S]-labeling, a considerable increase in yield of 8-aza-MPR was achieved by introduction of small changes in the previously reported procedures. The outline of the synthetic route is shown in Figure 1 and the details are given below.

3,6-Dihydro-3-β-D-ribofuranosyl-7H-1,2,3-triazolo[4,5-d]pyrimidine-7-thione (8-Aza-MPR). A solution of 7-(methylthio)-3-(2,3,5-tri-*O*-acetyl-β-D-ribofuranosyl)-3*H*-1,2,3-triazolo[4,5-*d*]pyrimidine (4) (3.11 g, 7.12 mmoles) in EtOH (112 ml) containing Fisher sodium hydrosulfide (5.57 g, powdered under EtOH) was stirred in a stoppered flask for 64 hr. The mixture was filtered and the precipitate washed with EtOH. The filtrate and wash were evaporated to near dryness at 25° *in vacuo*, and the residue was dissolved in EtOH (100 ml) containing NaOMe (1.54 g, 28.5 mmoles) and stirred for 20 hr. The mixture was evaporated to dryness at 25° *in vacuo*, and a solution of the residue in cold H_2O (48 ml) was filtered, adjusted to pH 7.2 with 6 N HCl, filtered and further adjusted to pH 6.5. The mixture was cooled in an ice bath and the white crystalline 8-aza-MPR collected, washed with cold H_2O and dried in vacuo (P_2O_5); yield 1.55 g (72%), mp 126° [resolidifies and melts at 189°, Kofler Heizbank (lit. (10) mp 128°)].

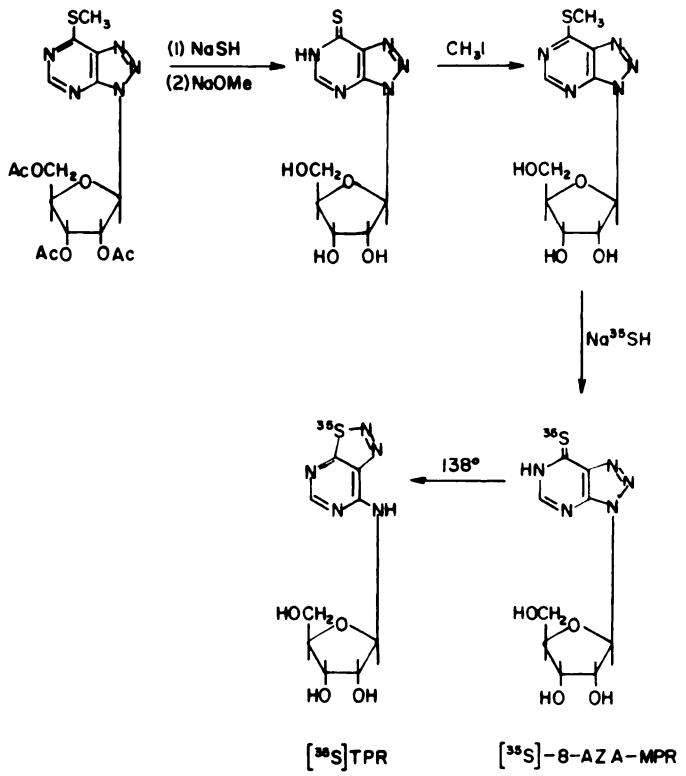

FIG. 1. *Scheme for the synthesis of [^{35}S]-8-aza-MPR and [^{35}S]TPR*

Anal. Calcd for $C_9H_{11}N_5O_4S \cdot H_2O$: C, 35.64; H, 4.32; N, 23.09. Found: C, 35.77; H, 4.22; N, 23.05.

7-(Methylthio)-3-β-D-ribofuranosyl-3H-1,2,3-triazolo[4,5-d]pyrimidine (8-Aza-MeMPR). A mixture of 8-aza-MPR (200 mg, 0.660 mmole) and $NaHCO_3$ (52.9 mg, 0.629 mmole) in H_2O (2 ml) was cooled in an ice bath, treated with MeI (0.047 ml, 0.755 mmole) and stirred in a tightly stoppered vial in an ice bath for 1 hr. The mixture was then shaken vigorously at 25° for 2 hr, stirred for 18 hr, and cooled in an ice bath. The precipitate of 8-aza-MeMPR was collected, washed with cold H_2O and dried *in vacuo* (P_2O_5); yield 170 mg (86%), mp 149° (Kofler Heizbank) (lit. (4) mp 150°).

[^{35}S]-8-Aza-MPR. A solution of $Na^{35}SH$ (28 mg, 0.50 mmole, 22.4 mCi) (Amersham, Arlington Heights, IL) in H_2O (9 ml) was stirred in a stoppered vial with 8-aza-MeMPR (75.0 mg, 0.250 mmole) for 3 hr. This solution was treated with pure 8-aza-MPR (200 mg, 0.660 mmole) and 0.33 N NaOH (1.5 ml) and stirred vigorously until most of the added 8-aza-MPR had dissolved (about 30 min). The solution was filtered under N_2 pressure, cooled in an ice bath and taken to pH 6.5–7.0 by addition of 6 N HCl. The mixture was stirred in the ice bath for 10 min and the precipitate was collected by pressure filtration under N_2, washed with cold H_2O (~3 ml) and dried *in vacuo* over P_2O_5. The product (221 mg), which contained elemental sulfur, was stirred vigorously for 1 hr with benzene (70 ml), collected by filtration and dried *in vacuo* over P_2O_5; yield 210 mg (76%), mp 126°. The above reaction was carried out in a glove box. Evolved $H_2{}^{35}S$ was trapped by drawing a slow stream of air through the box into a Clorox trap using water respirator vacuum. The specific activity was 43.1 μCi/mg and the radiochemical purity was 96%, as determined by scans of thin layer chromatograms on Analtech Silica Gel G developed with chloroform-methanol (5:1 v/v).

[^{35}S]TPR. For preparation of [^{35}S]TPR, a vial containing [^{35}S]-8-aza-MPR (95.2 mg, 0.314 mmole) was heated in an oil bath at 138° for 15 min; yield 91.9 mg (100%), mp 187°. The specific activity was 47.3 μCi/mg; and the radiochemical purity was >97% as determined by TLC chromatography as described above. The increased specific activity over that of the starting material is a result of loss of H_2O of hydration of the starting material.

Other materials. 8-Azainosine (11), and [2-^{14}C]-8-azaguanine (12) were synthesized in our laboratories. The following labeled compounds were obtained from New England Nuclear (Boston, MA); [2-^{14}C]-thymidine; [2-^{14}C]uridine; [1-^{14}C]-L-leucine; and [γ-^{32}P]ATP. Crystalline PNP [22 units/mg protein] from calf spleen, alkaline phosphatase [28 units/mg protein] from *E. coli*, and 5′-nucleotidase [35 units/mg protein] from *Crotalus adamanteus* were obtained from Sigma Chemical Co., St. Louis, Mo.

Cell culture and cell culture assays.

H.Ep. #2 cells were grown as monolayers or in suspension cultures in SRI 14 medium (13). Cytotoxicities of candidate compounds were determined by effects on colony formation as described in Table 1. Studies of the effectiveness of metabolites in reversing the cytotoxicities of candidate compounds were also accomplished by colony counts, as described in Fig. 9. Leukemia L1210 cells were grown in suspension culture in Fischer's medium (14). Effects of 8-aza-MPR and TPR on synthesis of DNA, RNA, and protein were determined by their effects on the utilization of [^{14}C]-labeled thymidine, uridine and leucine; the procedure used was a modification (15) of that described by Hershko *et al.* (16).

Assays of nucleosides as substrates for adenosine kinase. Nucleosides were assayed as substrates for adenosine kinase present in high speed supernatants from H.Ep. #2 cells or from adenosine kinase partially purified from H.Ep. #2 cells as previously described (17). The incubation mixture contained ATP, 2.5 μmoles; $MgCl_2$ 0.25 μmole; nucleoside at various concentrations, and enzyme in a final volume of 1 ml in phosphate buffer, pH 7.0. Incubation was at 25° for 30 min. In experiments in which the nucleosides were not labeled, the reaction mixture was the same except that [γ-^{32}P]ATP was substituted for ATP. The reaction was stopped by heating in a boiling water bath for 1 min, and a portion of the mixture was subjected to chromatography on a strip of Whatman 3M paper using a solvent consisting of equal parts of 93.4% aqueous 1-butanol and 44% aqueous propionic acid. The chromatograms were scanned with a Packard model 7201 chromatogram scanner to determine the positions and [^{35}S]-content of the radioactive compounds present. These procedures are described elsewhere (17).

Identification of the kinase responsible for phosphorylation of TPR. To determine the identity of the kinase catalyzing the phosphorylation of TPR, competitive studies were carried out with [^{35}S]TPR and MeMPR in crude supernatants from H.Ep. #2 cells. MeMPR is a known substrate for adenosine kinase and is preferred over adenosine to determine adenosine kinase activity in crude systems because it is not subject to degradation (17–19). The conditions of the assays were those described above for adenosine kinase except that MeMPR was present at various concentrations. Additional details are given in Figure 2.

Substrate activities for PNP or nucleoside hydrolases. [^{35}S]Labeled 8-aza-MPR and TPR were evaluated as substrates for PNP from calf spleen; [^{14}C]labeled inosine and adenosine were used as control substrates, inosine as a control substrate that is readily cleaved, and adenosine as a control substrate that is very poorly or not at all cleaved by this enzyme. The incubation mixtures contained in a total volume of 1 ml PNP (0.22, 1.1, 5.5, or 27.5 units), nucleoside, (1 μmole), and 0.1 M phosphate buffer, pH 7.4. Incubation was at 25°; 0.2 ml portions of the incubation mixture were removed at 1, 2, 5, 10, and 20 min. The reaction was stopped by the addition of 0.3 ml ethanol and the mixture was chromatographed on Whatman 3M paper using the butanol-propionic acid solvent described above. The paper strips were scanned in a Packard Model 7201 scanner. Assays of nucleosides as substrates for nucleoside hy-

TABLE 1

Cytotoxicities to H.Ep. #2 cells of 8-aza-MPR, TPR, and their aglycones

One hundred cells were placed in 4 oz prescription bottles in 10 ml of SRI-14 medium (13) (controls) or in 10 ml of medium containing the candidate inhibitors at various concentrations. After the cultures had been incubated at 37° for 7–10 days, the medium was decanted and the cells adhering to the glass were washed with phosphate-buffered saline (0.85%), fixed with Bouin's fixative, and stained with Giemsa stain. Each value in the table represents the results of two or more experiments. Each experiment had 8 controls; the cloning efficiency of controls varied considerably between separate experiments (range 40–70 colonies/100 cells) but was reproducible within a given experiment (standard deviations, ±12%).

	Concentration (μM) required for 50% inhibition of colony formation
8-Aza-MPR	1.8
TPR	0.14
8-Aza-MP	40
TP	>160

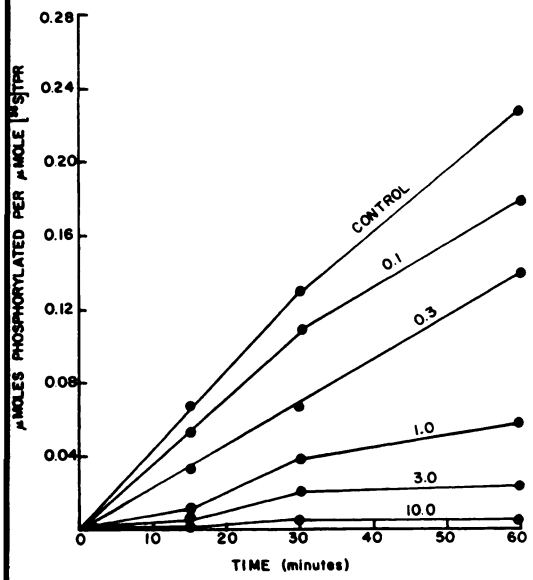

FIG. 2. *Effects of MeMPR on the phosphorylation of TPR in supernatants from cultured H.Ep. #2 cells*

The assays were performed with [^{35}S]TPR, unlabeled MeMPR and 20,000 × *g* supernatants from H.Ep. #2 cells as is described in detail in the text. Control tubes contained [^{35}S]TPR (1.0 μmole/ml, 5.5 μCi/ml), enzyme and the incubation mixture; treated tubes contained in addition MeMPR at the concentrations (μmoles/ml) indicated on each curve.

drolases present in H.Ep. #2 cells were performed simultaneously with the assays as substrates for adenosine kinase (see above). Crude supernatants from H.Ep. #2 cells were incubated with [^{35}S]-labeled TPR and 8-aza-MPR under the conditions described for the assay of adenosine kinase, and the products were assayed by chromatography on Whatman 3M paper with the butanol-propionic acid solvent. This solvent gives a good separation of bases, nucleosides, and nucleotides so that any conversion of 8-aza-MPR or TPR to the aglycones would be readily detectable.

Metabolism of 8-Aza-MPR and TPR. To determine possible incorporation of 8-aza-MPR and TPR into polynucleotides, [^{35}S]-8-aza-MPR and [^{35}S]TPR were added to 1 liter suspension cultures of proliferating H.Ep. #2 cells (~4.5 × 10^5 cells/ml). After incubation for 24 hr at 37° the cells were harvested and washed free of medium with 0.85% saline. The cells were then extracted with cold 0.5 N $HClO_4$ (1 ml/2.5 × 10^8 cells) and the $HClO_4$ extract was used for analysis by high pressure liquid chromatography (HPLC) as described below; these procedures have been described in detail elsewhere (15). From the residue remaining after the $HClO_4$ extraction, a crude polynucleotide fraction was isolated by extraction with hot 10% NaCl solution. This fraction was analyzed for polynucleotide content by determination of uv absorption at 260 nm and the [^{35}S]-content was determined with a Packard liquid scintillation spectrometer.

Loss of sulfur from 8-aza-MPR or its phosphate would lead to 8-azainosine or 8-azaIMP, and this conversion would of course not be detected in experiments with [^{35}S]-8-aza-MPR. Because 8-azainosine is itself cytotoxic (20), any conversion of 8-aza-MPR to 8-azainosine would contribute to the biological activity of 8-aza-MPR; therefore it was desirable to determine if this conversion occurred. Since [^{14}C]-8-aza-MPR was not available, it was necessary to attempt to accomplish this measurement by means other than the use of radioisotope-labeling. The principal soluble metabolite of 8-azainosine is 8-azaGTP (20), and a measure of any 8-azaGTP formation would therefore be a means of estimating the extent of desulfuration of 8-aza-MPR or its phosphate. It was first necessary to establish conditions for identifying 8-azaGTP in the presence of the natural nucleotides. A preliminary experiment was performed in which H.Ep. #2 cells were grown in the presence of [2-^{14}C]-8-azaguanine, after which the cells were extracted with 0.5 N $HClO_4$ and subjected to HPLC analysis on a Partisil-10 SAX column as described below. Fractions of 1 ml were collected and assayed for radioactivity. Radioactivity was found in a peak that eluted just after GTP and that was also detectable by monitoring at 254 nm. The fact that 8-azaGTP is formed from 8-azainosine and that it is easily separated from the natural nucleotides provided the basis for determining the extent of desulfuration of 8-aza-MPR.

In other experiments designed to study the soluble metabolites of these nucleosides, unlabeled precursors were used. The

H.Ep. #2 cells were grown as described above and 8-aza-MPR or TPR were added at various concentrations. The cells were harvested at various times thereafter, and a $HC10_4$ extract was prepared and used for analysis by HPLC; such analysis provided in the same experiment a means of isolating metabolites of the precursors and determining the effects on pools of natural nucleotides.

HPLC analysis. Analysis by HPLC was performed at ambient temperature with a Waters Associates (Milford, MA) model 202 apparatus and either a Partisil-10 SAX ion exchange column (Whatman, Inc., Clifton, NJ), 4.6 mm i.d. × 25 cm, or a reverse phase μ Bondapak C_{18} column (Waters) 3.9 mm i.d. × 30 cm. For ion exchange chromatography a linear (40 min) gradient from 5 mM $NH_4H_2PO_4$, pH 2.8, to 750 mM $NH_4H_2PO_4$, pH 3.7, was used. The flow rate was 2 ml/min and detection of eluted materials was accomplished by measurement of uv absorbance at 254, 280, 325 or 340 nm. Integrations of peak areas were made with a Hewlett-Packard model 3380-A digital electronic integrator, and quantitations for ribonucleotide pool studies were achieved using peak area-nmole relationships derived from determination of known nucleotides. For reverse phase chromatography, resolution was achieved with a solvent consisting of H_2O:acetic acid:dioxane (1000:15:20 (v/v/v)). Detection of peaks was accomplished as described above for ion exchange chromatography. When radioactive precursors were used 1 ml fractions were collected for assay in a Packard liquid scintillation spectrometer.

Both 8-aza-MPR and TPR have significant absorption at wave lengths above 300 nm whereas the natural nucleotides do not. It was therefore possible, by monitoring the elution simultaneously at 254, 280, and 340 nm, to measure the metabolites of the thio compounds in the presence of the natural nucleotides and at the same time to determine the pool sizes of the natural nucleotides. The absorption spectra of 8-azaMPR and TPR are sufficiently different that they can be used to identify metabolites: at pH 1 8-aza-MPR has a single maximum at 328 nm whereas TPR has maxima at 244, 267-272 (a broad peak), and 306 nm (4). Peaks were scanned during elution by the use of a Beckman stop-scan spectrophotometer.

Synthetic samples of the phosphates of 8-aza-MPR and TPR were not available for use in establishing quantitative relationships between areas of peaks eluted from the Partisil column and concentrations of the nucleotides. In the absence of known samples of the nucleotides, it was assumed that the relationships between peak areas and nucleotide concentration for the ion exchange column would be the same as the relationships between peak areas and concentrations experimentally determined with known samples of the corresponding nucleosides eluted from the reverse phase column.

RESULTS

Cytotoxicity of 8-Aza-MPR, TPR, and their aglycones (Table 1). Both 8-aza-MPR and TPR were toxic to cultured H.Ep. #2 cells; the ED_{50} (concentration required for 50% inhibition of colony formation) of TPR was about 10-fold lower than that of 8-aza-MPR. The aglycones of these nucleosides were less toxic; the ED_{50} for 8-aza-MP was 40 μM and TP was not toxic at the highest concentration assayed (160 μM).

TPR as a substrate for adenosine kinase. To determine if TPR was phosphorylated, [^{35}S]TPR was incubated with crude supernatants from H.Ep. #2 cells under the conditions described above for assay of adenosine kinase. There was extensive conversion of TPR to a compound with the properties of a monophosphate as shown by both paper chromatography and HPLC on a Partisil-10 SAX column (results not shown). The addition of MeMPR to the incubation mixture produced a concentration-dependent decrease in the phosphorylation of [^{35}S]TPR (Fig. 2); this result suggests that AK catalyzes the phosphorylation of TPR. TPR was accordingly evaluated as a substrate for AK 135-fold purified from H.Ep. #2 cells, with the results shown in Figure 3. The observed kinetic constants for TPR are compared with those of related substrates in Table 2. 8-Aza-MPR and TPR had about the same V_{max}, but the K_m for TPR was lower than that for 8-aza-MPR.

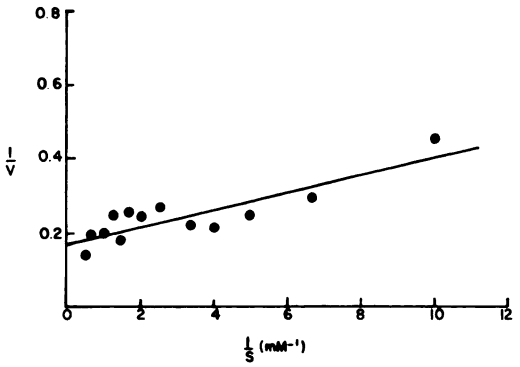

FIG. 3. *Lineweaver-Burk plot for the phosphorylation of TPR by adenosine kinase*

The enzyme was 135-fold purified from cultured H.Ep. #2 cells. Assays were performed as described in the text using unlabeled TPR and [γ-^{32}P]ATP. Values for the ordinates are the reciprocals of the reaction rate in nmoles/min.

TABLE 2

Comparison of 8-aza-MPR, TPR and some related compounds as substrates for adenosine kinase

The assays were performed as described in the text with enzyme preparations purified to varying degrees from cultured H.Ep. #2 cells. Kinetic constants for the substrates other than TPR and 8-aza-MPR have been reported earlier (3, 17), and are listed here for direct comparison with TPR.

Substrate	Kinetic constants for phosphorylation by adenosine kinase	
	K_m	V_{max} (relative to adenosine)
	μM	
Adenosine	1.8	100
MeMPR	50	330
8-Aza-MPR	560	90
TPR	132	108
MPR	—[a]	—

[a] No activity for the substrate could be detected under the conditions of the standard assay.

The lack of any stimulatory effect of pH on the phosphorylation of TPR is shown in Figure 4. This lack of effect is in marked contrast to the effect of pH on the phosphorylation of 8-aza-MPR, which we have determined previously (3), but which was redetermined for direct comparison with TPR.

Substrate activities for PNP or nucleoside hydrolases. When [^{35}S]labeled 8-aza-MPR and TPR were examined as substrates for PNP from calf spleen, there was no detectable formation of either of the free bases. Under the same conditions, as expected, inosine was extensively converted to hypoxanthine and there was no detectable conversion of adenosine to adenine. That 8-aza-MPR and TPR are also not substrates for any nucleoside hydrolases occurring in H.Ep. #2 cells was shown in experiments in which the metabolism of these substrates was examined in crude supernatants; no free bases were detected. These negative results are not shown.

Failure of [^{35}S]-8-aza-MPR and [^{35}S]-TPR to be incorporated into polynucleotides. To determine if 8-aza-MPR or TPR were incorporated into polynucleotides, 1 liter cultures of H.Ep. #2 cells (~2.5 × 10^8 cells) were grown for 24 hrs in the presence of [^{35}S]-8-aza-MPR (43 μCi/flask; 1 μg/ml) or [^{35}S]TPR (47 μCi/flask; 1 μg/ml) as described in MATERIALS AND METHODS. The crude polynucleotide fractions for experiments with either precursor had a total [^{35}S]-content of the order of 10^{-3} μCi. When

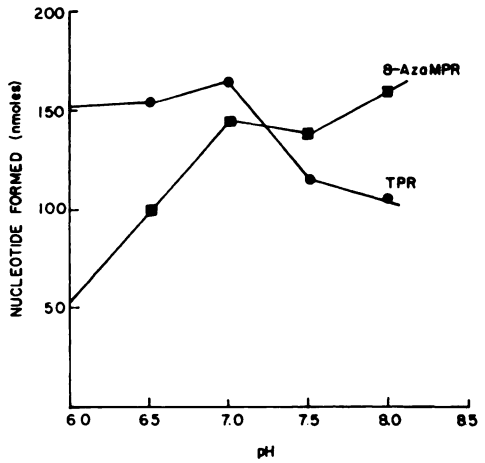

FIG. 4. *Effect of pH on the phosphorylation of TPR and 8-aza-MPR*

The incubation mixture contained the standard constituents for the assay of AK (see text); enzyme (20,000 × g supernatant from H.Ep. #2 cells), 2.2 mg; and [^{35}S]-8-aza-MPR or [^{35}S]TPR (1.0 μmole/ml, 5.5 μCi/ml), in phosphate buffer of pH ranging from 6–8. After 30 min incubation the reaction was stopped by addition of two volumes of methanol and the nucleotides formed were isolated by paper chromatography. See text for details.

DNA and RNA were separated by the Schmidt-Thannhauser procedure (21), there was no detectable [^{35}S] in either the DNA or in the ribonucleotide fraction resulting from hydrolysis of RNA. This result indicates that the small amounts of [^{35}S] found in the crude polynucleotide fraction does not represent incorporation of a [^{35}S]labeled metabolite into the polymer, but rather an association or binding to this fraction of [^{35}S] in a form that is volatilized under the conditions of the Schmidt-Thannhauser procedure.

Soluble metabolites of 8-Aza-MPR and TPR. When H.Ep. #2 cells were grown in the presence of [^{35}S]labeled 8-aza-MPR or TPR and the soluble metabolites analyzed by HPLC, radioactivity was found only in compounds with a retention time in the range of those of the natural nucleoside monophosphates and corresponding most closely to the retention time of AMP (results not shown). Further analysis of the soluble metabolites was performed with unlabeled substrates because better resolution was obtained by monitoring of uv absorption than could be obtained by analysis of [^{35}S] content. The results are shown in Figure 5. Three metabolites with retention times characteristic of monophosphates were obtained from 8-aza-MPR (Fig. 5a). When each of the nucleotide peaks were scanned with a Beckman stop-scan spectrophotometer, the major peak was found to have absorption characteristic of 8-aza-MPR and those of the two smaller peaks were characteristic of TPR. For further identification of these metabolites, portions of the cell extracts were treated overnight at 37° with alkaline phosphatase in tris buffer, pH 8.0, or with 5′-nucleotidase in glycylglycine buffer, pH 9.0, after which the reaction was stopped by immersion of the flasks in a boiling water bath. When this mixture was chromatographed on a reverse phase column and elution was monitored at 340 nm, three principal peaks (Fig. 5c) were detected, of which one corresponded in retention time and uv absorption spectrum to 8-aza-MPR, one to the β-anomer of TPR, and one to the α-anomer of TPR, as established in separate experiments in which known samples of 8-aza-MPR and the α- and β-anomers of TPR were chromatographed singly and as a mixture. In the course of these experiments it was found that the short period of heating used to stop the phosphatase reaction caused significant rearrangement of 8-aza-MPR to TPR, and also produced α-β isomerization

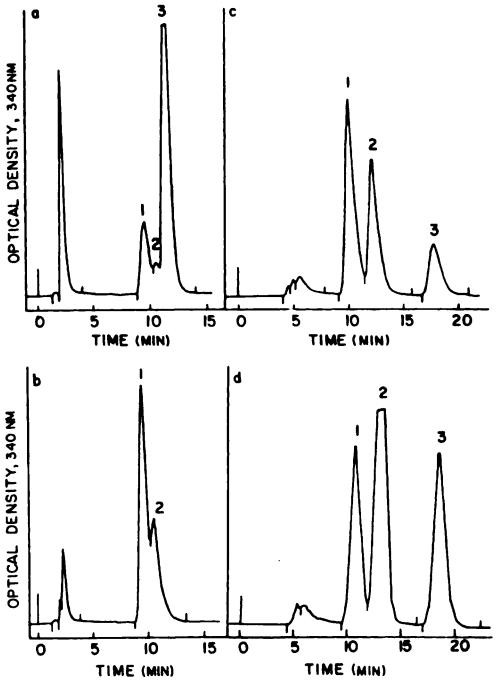

FIG. 5. *Analysis by HPLC of extracts of H.Ep. #2 cells grown in the presence of 8-aza-MPR or TPR*

To proliferating cultures of H.Ep. #2 cells were added 8-aza-MPR or TPR, each at a concentration of 35 μM; the cells were harvested 24 hrs thereafter and a 0.5 N $HClO_4$ extract prepared for HPLC analysis or an ion exchange column (panels a and b). Portions of the cell extract were treated overnight with alkaline phosphatase and chromatographed on a μC_{18} reverse phase column (panels c and d). a) 8-Aza-MPR as precursor; ion exchange column; b) TPR as precursor; ion exchange column; c) 8-aza-MPR as precursor; reverse phase column; d) TPR as precursor, reverse phase column. The uv absorption spectrum was determined during elution for each peak by use of a Beckman stop-scan spectrophotometer. The identities established for each peak were: Chart a: 1, β-TPR phosphate; 2, α-TPR phosphate; 3, 8-aza-MPR phosphate. Chart b: 1, β-TPR phosphate; 2, α-TPR phosphate. Charts c and d: 1, 8-aza-MPR, 2, β-TPR, 3, α-TPR. The unnumbered peaks in Charts a and b with retention times of 1–2 min are nucleosides, bases and any other nonpolar compounds not retained on the column. See text for further details.

of TPR. Therefore, the amounts of nucleosides isolated on the reverse phase column do not correspond quantitatively to the nucleotides isolated on the ion exchange column. The results of the reverse phase chromatography do provide additional evidence for the identity of the three metabolites as the monophosphates of 8-aza-MPR, of the β-anomer of TPR, and of the α-anomer of TPR. Further, the conversion of these nucleotides to nucleosides by the action of 5′-nucleotidase is evidence that the nucleotides have their phosphate in the 5′-position.

When TPR was the precursor, two compounds with retention times characteristic of monophosphates were found upon chromatography on the ion exchange column (Fig. 5b). Both of these gave uv scans characteristic of TPR. When the cell extract was treated with alkaline phosphatase or 5′-nucleotidase and chromatographed on the reverse phase column, as described above, three peaks were obtained (Fig. 5d), two of which gave uv scans characteristic of TPR and the other of which gave a scan characteristic of 8-aza-MPR. The presence of 8-aza-MPR was shown to be an artifact of the heating incident to the alkaline phosphatase procedure; in a separate experiment it was found that heating a solution of TPR for 2 min at 100° in the buffer used for the alkaline phosphatase treatment produced a rearrangement of 28% of the TPR to 8-aza-MPR. This was unexpected because at 23° in aqueous solutions at various pHs, no rearrangement of TPR to 8-aza-MPR occurred over a period of several days, whereas 8-aza-MPR rearranged slowly to TPR (results not shown). Thus it appears that the short periods of heating in the buffer used for the phosphatase reaction can cause interconversion of 8-aza-MPR and TPR, whereas in aqueous solution at room temperature or in the solid phase at 138° the conversion of 8-aza-MPR to TPR is not reversible. Rearrangement during isolation does not contribute to the nucleotide profiles shown in Figure 5 because these profiles represent chromatograms of cold perchloric extracts which were kept frozen until the application to the ion exchange column. The quantitation of the nucleotides (see below) is based on these chromatograms. Rearrangement during heating does affect the amounts of nucleosides isolated on reverse phase columns after treatment of the cell extract with phosphatase; reverse phase chromatography was used only to confirm identifications and not for quantitation. It should be noted that the sizes of the peaks in Figure 5 are not indicative of the relative amounts of each nucleotide or nucleoside present; this is because elution was monitored at 340 nm, a wave length at which 8-aza-MPR and its phosphate absorb much more strongly than do TPR and its phosphate. Thus in Figure 5a peak 1 represents more nucleotide (β-TPR phosphate) than peak 3 (8-aza-MPR phosphate).

The concentrations of the analogue nucleotides found in H.Ep. #2 cells are shown in Table 3. The total amount of nucleotides derived from TPR was 10–20 fold greater than that derived from 8-aza-MPR. When

TABLE 3

Concentrations of phosphates of 8-aza-MPR and TPR in H.Ep. #2 cells

H.Ep. #2 cells in 1 l. suspension cultures (~5 × 10^5 cells/ml) were grown for 4 or 24 hr in the presence of 8-aza-MPR or TPR each at a concentration of 35 μM, after which the nucleotide content of the cells was analyzed by HPLC as described in the text.

Precursor Added	Time (hr)	nmoles/10^9 cells		
		8-aza-MPR phosphate	β-TPR phosphate	α-TPR phosphate
8-aza-MPR	4	67	68	——[a]
8-Aza-MPR	24	88	158	——[a]
TPR	4		2191	756
TPR	24		1527	872

[a] α-TPR phosphate was present but at concentrations too low for quantitation.

8-aza-MPR was the precursor, the phosphate of β-TPR was present at a concentration equal to that of the phosphate of 8-aza-MPR at 4 hr and about twice as great at 24 hr. When TPR was the precursor the α-anomer of TPR-phosphate was present at 30–50% of the concentration of the β-anomer. The total concentration of phosphates of TPR is about half that of the concentration of ATP found in control cultures of H.Ep. #2 cells. The results shown in Table 3 are those of a single experiment. In another experiment performed under identical conditions the amount of nucleotides derived from TPR was about the same as that of the first experiment, whereas the amount of nucleotides from 8-aza-MPR was about twice that of the first experiment. These differences may reflect difficulty in quantitation of the smaller amounts of nucleotides derived from 8-aza-MPR or differences in the metabolic state of the cells used in two experiments.

Comparison of the metabolism of 8-Aza-MPR and 8-azainosine and their effects on nucleotide pools. As discussed under MATERIALS AND METHODS 8-azaGTP is the principal soluble metabolite of 8-azainosine and therefore its formation affords a means of detecting desulfuration of 8-aza-MPR or its phosphate. The formation of 8-azaGTP from 8-azainosine is shown in Figure 6. This peak is absent from cells grown in the presence of an equimolar concentration of 8-

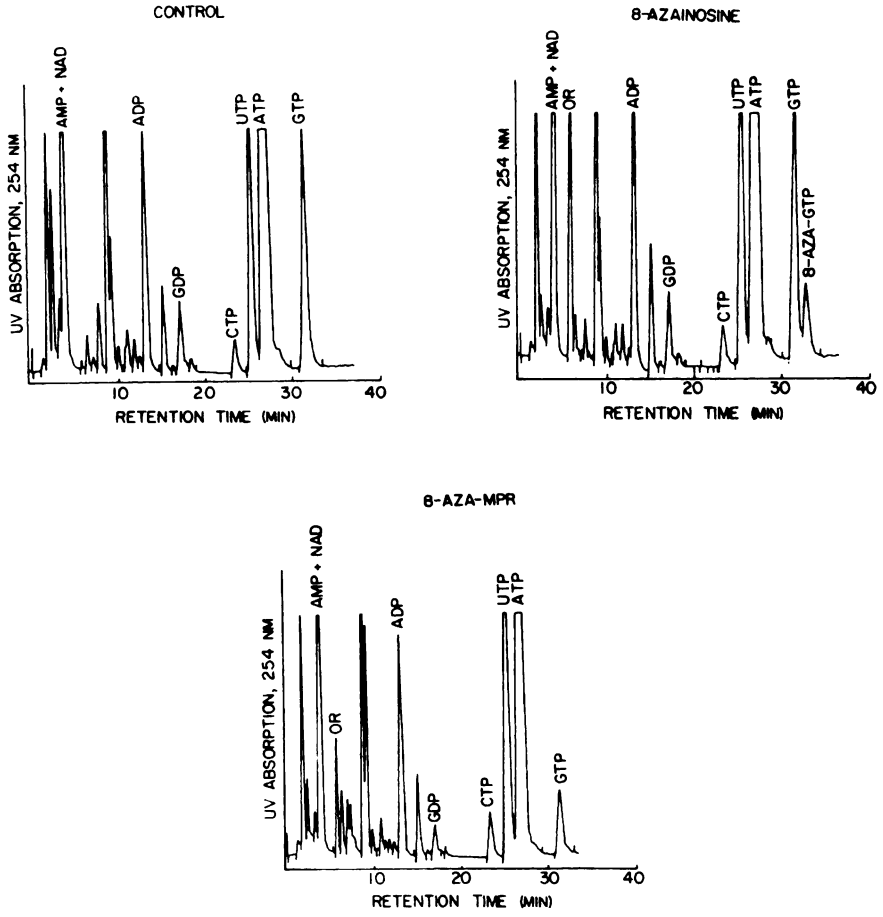

FIG. 6. *Evidence for the failure of 8-aza-MPR to be converted to 8-azainosine or to 8-azaGTP*

To proliferating suspension cultures of H.Ep.#2 cells were added 8-azainosine or 8-aza-MPR each at a concentration of 10 μM; control cultures received no additions. After 4 hr the cells were harvested and extracts were prepared and analyzed by HPLC as described in the text. OR, orotidine.

aza-MPR, and was also absent from an independent experiment in which a higher concentration (18 μM) of 8-aza-MPR was used.

In cells treated with either 8-azainosine or 8-aza-MPR a new peak appeared in the monophosphate region with a retention time of about 6 min. This peak has the retention time and the uv absorption spectrum (as determined by stop-scan measurements) of orotidine. Its identity was further established by a "reinforcement" experiment, that is, an experiment in which orotidine was added to the cell extract before chromatography; the suspected orotidine peak was increased with no effect on any other peaks. Because inhibition of pyrimidine synthesis with a consequent accumulation of orotidine is a known effect of 8-azainosine (20), no further identification of this peak was attempted.

A notable effect of 8-aza-MPR in this experiment was the reduction of the GTP pool, an effect not produced by 8-azainosine. A more detailed analysis of effects on nucleotide pools is given below.

Effects of 8-aza-MPR and TPR on pool sizes of ribonucleotides. Both 8-aza-MPR and TPR reduced the pools of purine ribonucleotides with little effect on those of pyrimidine nucleotides. The effect on the guanine nucleotide pool was consistently much greater than that on the adenine nucleotides (Figs. 6 and 7). The results shown are for the combined mono-, di-, and triphosphates of adenosine; the combined di- and triphosphates of guanosine; and for the triphosphates of uridine and cytidine. The adenine nucleotides also include NAD, which is not well separated from AMP. GMP, UMP, CMP, CDP, and UDP are not included in the totals either because of their small pool sizes or incomplete resolution; UTP and CTP represent almost all of the pools of the pyrimidine nucleotides. Similar effects of the inhibitors were observed on the various phosphates of a given nucleoside; for example when depression of the pool of adenine nucleotides was observed, essentially equal decreases occurred in AMP, ADP, and ATP. Figure 7 shows pool sizes as percentages of controls; the absolute values (nmoles/10^9 cells) for control

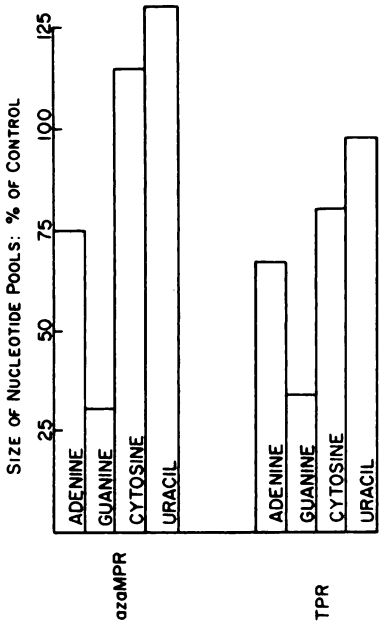

FIG. 7. *Effects of 8-aza-MPR and TPR on the ribonucleotide pools of H.Ep. #2 cells*

H.Ep. #2 cells were grown in suspension culture for 4 hr in the presence of 8-aza-MPR (10.5 μM) or TPR (1.8 μM); control cultures received no additions. Extracts of the cells were analyzed by HPLC as described in the text; see also Figure 6. The compositions of the nucleotide pools shown are: adenine, AMP + NAD + ADP + ATP; guanine, GDP + GTP; cytosine, CTP: uracil, UTP (see text for explanation).

cultures of H.Ep. #2 cells for these and other experiments fell in the following ranges: adenine, 5,000–7,000; guanine, 800–1,100; cytosine, 200–300; uracil 1,200–2,000. High concentrations of inhibitor and longer periods of exposure did not depress further the guanine nucleotide pools but did depress strongly the pools of nucleotides of adenine, uracil, and cytosine (results not shown).

Effects on synthesis of macromolecules. Both 8-aza-MPR and TPR inhibited incorporation of thymidine and uridine and were without effect on incorporation of leucine (Fig. 8).

Reversal studies (Fig. 9). As determined by colony counts with H.Ep. #2 cells, the cytotoxicity of TPR was prevented in part or totally by AIC, hypoxanthine, adenine, or by combinations of adenine and guanosine and hypoxanthine and uridine. In con-

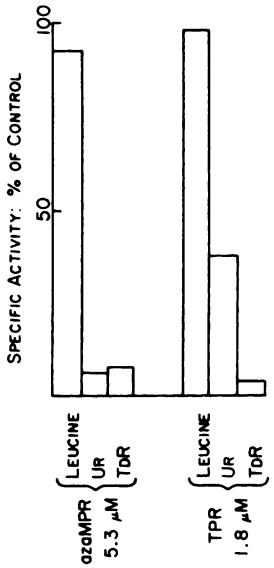

FIG. 8. *Inhibition of macromolecular synthesis in H.Ep. #2 cells by 8-aza-MPR and TPR*

To H.Ep.#2 cells in suspension culture were added 8-aza-MPR (5.3 μM) or TPR (1.8 μM); control cultures received no additions. One hour thereafter one of the following [^{14}C]labeled precursors was added each at a final concentration of 0.01 μCi/ml and at the indicated μM concentrations: [2-^{14}C]thymidine, 0.17; [2-^{14}C]uridine, 0.19; [1-^{14}C]leucine, 0.20. Cells were harvested 4 hr after addition of the labeled precursors and radioactivity present in the macromolecular fraction was determined. The results shown are for triplicate experiments. The specific activities (nCi/mg) of the macromolecular fractions for control experiments were: [2-^{14}C]thymidine as precursor, 25.4; [2-^{14}C]uridine as precursor, 36.3; [1-^{14}C]leucine as precursor, 11.3. See text for additional details.

trast, no single agent was highly effective in preventing the cytotoxicity of 8-aza-MPR but a combination of hypoxanthine and uridine restored colony formation to about 75% that of controls.

DISCUSSION

This study was undertaken on the basis of the prediction that 8-aza-MPR, in contrast to MPR, would be readily phosphorylated and not subject to degradation by PNP; it was also expected that 8-aza-MPR phosphate would have biological activity similar to that of MPR phosphate. If these predictions were realized, then 8-aza-MPR might have some advantages over MP or MPR as an antileukemic agent. Although 8-aza-MPR was, as predicted, a good substrate for AK and not a substrate for PNP, it did not show consistent activity against L1210 leukemia in mice, whereas its rearrangement product, TPR, had antileukemic activity about equivalent to that of MP (F. M. Schabel, Jr., and W. R. Laster, Jr., Southern Research Institute, unpublished results). The dissimilarity in antileukemic activity of MPR and 8-aza-MPR probably is due to the ease of rearrangement of 8-aza-MPR and its phosphate to TPR and its phosphates with the result that after 24 hr exposure of H.Ep. #2 cells to 8-aza-MPR the phosphates of TPR are present in amounts equal to or greater than those of 8-aza-MPR phosphate (Table 3). The higher toxicity (Table 1) and the better antileukemic activity of TPR probably are the result of the fact that it is much more extensively converted to nucleotides than is 8-aza-MPR (Table 3). Perhaps the most interesting finding resulting from this investigation was the biological activity of

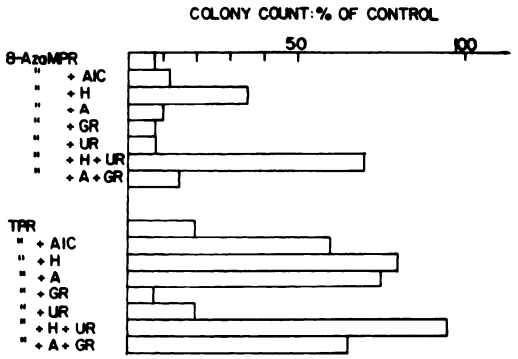

FIG. 9. *Effectiveness of selected compounds in reversing or preventing inhibition of H.Ep. #2 cells by 8-aza-MPR and TPR*

Toxicities were determined by the colony count method as described in Table 1. Each 4 oz. prescription bottle contained 100 cells in 10 ml of SRI-14 medium. Inhibitors and candidate reversal agents were added at the same time and colonies were counted 10 days later. Abbreviations: AIC, 4-amino-5-imidazolocarboxamide; H, hypoxanthine; A, adenine; GR, guanosine; UR, uridine. The concentrations (μM) used were 8-aza-MPR, 2.3; TPR, 0.16; AIC, 319; H, 147; A, 37; GR, 71; and UR, 82. Triplicate assays were made for each concentration. The results shown are for single experiments which were confirmed in independent experiments. The cloning efficiencies for control cultures were in the range 76–86%.

TPR. TPR represents a new structure among biologically active nucleoside analogues in that its ribosyl moiety is attached to a primary amino group instead of a heterocyclic ring.

Inhibition of synthesis of purine nucleotides appears to be a primary site of action of TPR as shown by the capacity of AIC or hypoxanthine to prevent the toxicity of TPR (Fig. 9) and by the selective reduction of purine nucleotide pools in cells treated with TPR (Fig. 7). 8-Aza-MPR apparently inhibits the synthesis of both purine and pyrimidine nucleotides, but some of the results are not consistent with an inhibition of pyrimidine synthesis. The best evidence for a blockade of both pathways by 8-aza-MPR is the fact that a combination of hypoxanthine and uridine was required to prevent its toxicity; that this effect was not the result of inhibition of uptake of 8-aza-MPR is shown by failure of uridine alone to prevent the cytotoxic effects (Fig. 9). Additional evidence for inhibition of pyrimidine synthesis is the accumulation of orotate and orotidine in 8-aza-MPR-treated cells (Fig. 6). Results not in accord with such inhibition are those of Figure 7, which show no decrease in pools of pyrimidines at concentrations of 8-aza-MPR that reduced pools of purine nucleotides. This apparent discrepancy may be the result of the differences in time of exposure to the inhibitor in the various types of experiments. Both 8-aza-MPR and TPR inhibited the incorporation of uridine and thymidine into macromolecules without effect on protein synthesis (Fig. 8); these inhibitions conceivably could result from inhibition of the transport of these precursors, but, when considered with the other data, probably indicate inhibition of RNA and DNA synthesis as a result of depletion of pools of nucleoside triphosphates. The lesion responsible for killing of cells by these agents is not defined by these data. However, since inhibition of DNA synthesis or function is the mechanism of cell kill by a number and variety of agents, it is possible that inhibition of DNA synthesis resulting from reduced pools of deoxynucleoside triphosphates is responsible for the toxicity of 8-aza-MPR and TPR.

The selective reduction of pools of guanine nucleotides by 8-aza-MPR and TPR (Figs. 6 and 7) is suggestive of a site of action on the IMP-GMP conversion. However, in H.Ep. #2 cells guanosine did not prevent inhibition by these nucleosides (Fig. 9).

Because of the ease of rearrangement of 8-aza-MPR to TPR and the ease of α-β isomerization of TPR and its phosphate, cells grown in the presence of 8-aza-MPR contain phosphates of 8-aza-MPR, α-TPR, and β-TPR, and cells grown in the presence of TPR contain the latter two nucleotides. The apparent routes of formation of these metabolites are shown in Figure 10. The fact that more than one potentially inhibitory nucleotide is derived from either precursor makes it difficult to ascribe the observed biochemical and biological activities to a particular nucleotide. The fact that TPR blocks synthesis of purines indicates that one or both phosphates of TPR inhibit purine synthesis; these same nucleotides are also formed from 8-aza-MPR and presumably are responsible for its inhibition of purine synthesis. Because the phosphate of 8-aza-MPR is present in cells exposed to 8-aza-MPR but not in cells exposed to TPR, any inhibition of pyrimidine biosynthesis in these cells can be ascribed to its presence. This conclusion is consistent with the fact that 8-azainosine, the O-analogue of 8-aza-MPR, produces similar effects on pyrimidine synthesis (Fig. 6). The possibility is not excluded that 8-aza-MPR phosphate also contributes to the inhibition of purine synthesis, but this is not considered likely because 8-azainosine does not inhibit this pathway at concentrations well above those inhibiting cell proliferation (20).

A remaining question is the contribution of the α-anomer of TPR phosphate to the biological activity of TPR. This question cannot be answered from the data at hand because, as a result of the ready anomerization of TPR, there is no apparent way to study the biological activity of the α- and β-anomers separately. The α-anomer is present in considerable quantity; in cells exposed to TPR it exceeds in quantity that of 8-aza-MPR phosphate in cells exposed to inhibitory quantities of 8-aza-MPR (Table 3). The route of its formation has not

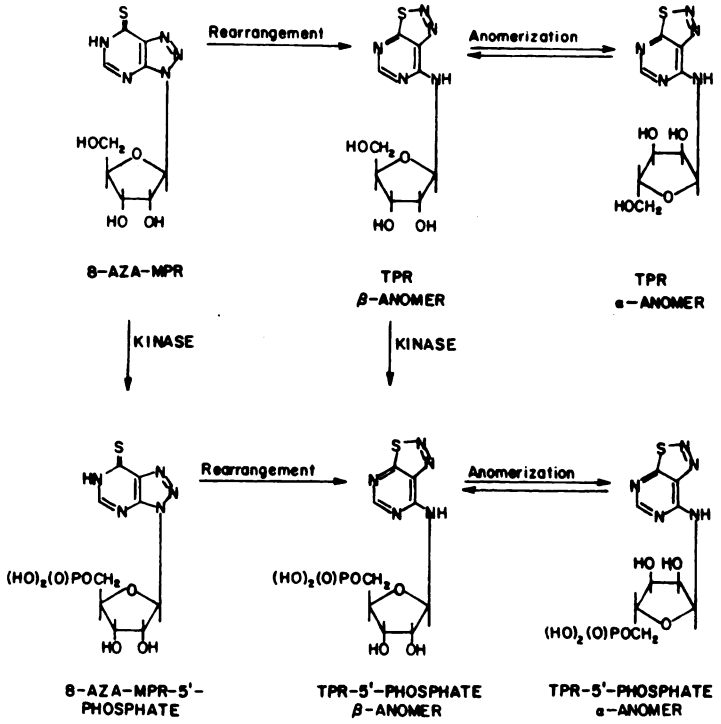

FIG. 10. *Pathways for the formation of the observed metabolites of 8-aza-MPR and TPR*

been proven, but it probably is formed by anomerization of β-TPR phosphate, since α-ribonucleosides (e.g., α-adenosine) do not have detectable activity as substrates for AK (22); evidence that α-TPR was not phosphorylated directly was obtained in an experiment that showed that the cytotoxicity of a mixture of α- and β-anomers of TPR was the same as that of the β-component alone (results not shown). That phosphates of α-nucleosides may have biological activity is known from studies with α-6-thiodeoxyguanosine (23, 24) and α-arabinosyl-8-azaadenine (25). With respect to the metabolism of TPR, it should be noted that isomerization of both anomers to the pyranoses also occurs readily (26). It is therefore likely that to some extent the pyranoses were formed in the cell culture medium or even intracellularly. If formed, the pyranoses probably would not contribute to the biological activity of TPR because it is unlikely that they would be phosphorylated by any of the known nucleoside kinases. The presence of phosphates of the pyranoses has not been rigorously excluded, but additional evidence that they are not present is the fact that the nucleotides derived from TPR were cleaved to nucleosides by the action of 5'-nucleotidase, which has specificity for ribo- and deoxyribonucleoside-5'-phosphates (27).

The failure of 8-aza-6-MPR to be incorporated into polynucleotides was unexpected. It was predicted that this compound would be incorporated either as 8-aza-6-thioguanine or 8-azaguanine. This prediction was based on the expectation that 8-aza-MPR should have properties in common both with MP, which is incorporated as 6-thioguanine (28, 29), and with 8-azainosine which is incorporated as 8-azaguanine (20). The absence of detectable [^{35}S] in polynucleotides from cells exposed to [^{35}S]-8-aza-MPR shows that there is little or no incorporation of this molecule in any form in which the S-atom is retained. Its incorporation as 8-azaguanine is not completely excluded, since [^{14}C]-8-aza-MPR was not available for study. However, the experiments shown in Figure 6 indicate that there is no extensive desulfurization of 8-

aza-MPR which would be readily apparent from the conversion of the product, 8-azainosine, to 8-azaGTP.

The fact that MeMPR, a known substrate for AK (17), competes with TPR for phosphorylation by kinases present in crude supernatants of H.Ep. #2 cells (Fig. 2) suggests that AK is the enzyme responsible for the phosphorylation of TPR. TPR is a good substrate for AK partially purified from H.Ep. #2 cells; the observed kinetic constants are of the same magnitude as those of MeMPR (Table 2). The phosphorylation of TPR is not pH-dependent in the range 6–8, in contrast to the phosphorylation of 8-aza-MPR which increases as the pH is raised over the range (Fig. 4); the behavior of TPR is similar to that of adenosine and MeMPR (3). The increased rate of phosphorylation of 8-aza-MPR as the pH is raised has been attributed to ionization of the N-1 proton (3); the behavior of TPR is consistent with this mechanism since, like MeMPR, it does not contain an N-1 proton. The activity of TPR as a substrate for AK was surprising; it is the first reported example of substrate activity by a nucleoside that does not have its pentofuranosyl group attached to a heterocyclic ring. The activity of TPR as a substrate may be rationalized by the fact that with respect to relative positions of the ribosyl moiety and the pyrimidine ring, TPR can assume any conformation possible for adenosine. Among the known substrates for AK, MeMPR most closely resembles TPR. In fact, TPR may be regarded as an analogue of MeMPR in which a N-atom of the thiadiazole ring replaces the methyl group and from which the 8-C atom has been removed. TPR and MeMPR also have biological properties in common: both have primary sites of inhibition on purine synthesis *de novo* and under normal conditions neither is metabolized to polyphosphates (30). These considerations indicate that biological activity might be found among other appropriately substituted 4-ribosylaminopyrimidines.

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