Biochemical Pharmacology, Vol. 34, No. 8, pp. 1293–1304, 1985.
Printed in Great Britain.

0006-2952/85 $3.00 +0.00

METABOLISM AND METABOLIC EFFECTS OF 2-AZAHYPOXANTHINE AND 2-AZAADENOSINE*

L. LEE BENNETT, JR.,† DONALD SMITHERS, LUCY M. ROSE, DORIS J. ADAMSON, SUE C. SHADDIX and H. JEANETTE THOMAS

Kettering-Meyer Laboratory, Southern Research Institute, Birmingham, AL 35255, U.S.A.

(*Received* 23 *April* 1984; *accepted* 20 *August* 1984)

Abstract—The metabolism and metabolic effects of 2-azahypoxanthine and 2-azaadenosine were studied to elucidate the biochemical basis for their known cytotoxicities. 2-Azaadenosine is a known substrate for adenosine kinase. That 2-azahypoxanthine is a substrate for hypoxanthine (guanine) phosphoribosyltransferase is shown by the observations that, in cell-free fractions from HEp-2 cells supplemented with 5-phosphoribosyl-1-pyrophosphate, (a) 2-azahypoxanthine inhibited the conversion of hypoxanthine to IMP but not the conversion of adenine to AMP, and (b) hypoxanthine, but not adenine, inhibited the conversion of 2-azahypoxanthine to 2-azaIMP. [8-^{14}C]2-Azahypoxanthine was synthesized from [8-^{14}C]hypoxanthine via [2-^{14}C]-4-amino-5-imidazolecarboxamide. In HEp-2 cells in culture, the principal metabolite of [8-^{14}C]-2-azahypoxanthine was 2-azaATP; there was no detectable ^{14}C in deoxynucleotides or in DNA or RNA fractions. 2-Azaadenosine was much more toxic than 2-azahypoxanthine, and, when used in the presence of an adenosine deaminase inhibitor, 2′-deoxycoformycin, was converted in HEp-2 cells to 2-azaATP in amounts that exceeded those of ATP in control cells. The pool of ATP was reduced by as much as 75% as 2-azaATP accumulated. In a short-term experiment (4 hr), 2-azaadenosine selectively reduced the pools of adenine nucleotides, whereas 2-azahypoxanthine reduced the pools of guanine nucleotides selectively. Both 2-azahypoxanthine and 2-azaadenosine inhibited the incorporation of formate into purine nucleotides and were without effect on the conversion of thymidine and uridine to nucleotides. 2-Azahypoxanthine inhibited the incorporation of thymidine into macromolecules but not that of uridine or leucine; 2-azaadenosine inhibited the incorporation of all three of these precursors non-selectively. 2-AzaIMP inhibited IMP dehydrogenase competitively with IMP (K_i= 66 μM). The difference in effects of 2-azahypoxanthine and 2-azaadenosine perhaps may be due to the production, from 2-azahypoxanthine but not from 2-azaadenosine + 2′-deoxycoformycin, of 2-azaIMP, which inhibits synthesis of guanine nucleotides and thereby results in inhibition of DNA synthesis. Specific sites of action for 2-azaadenosine are yet undefined.

2-Azahypoxanthine (2-azaHyp)‡, 2-azaadenine and their nucleosides are of interest as cytotoxic compounds of as yet undefined modes of action [1]. 2-AzaHyp has additional interest as a known product of decomposition of 5-(3,3-dimethyl-1-triazenyl)-1*H*-imidazole-4-carboxamide (DTIC) [2–4]; although 2-azaHyp is not responsible for the primary antitumor activity of DTIC, it may contribute to the biological effects of this important antitumor agent. A significant feature of the metabolism of 2-azaHyp and 2-azaadenosine (2-azaAdo) is that, unlike many other purine analogs, they obviously cannot be converted to analogs of guanine nucleotides. For this reason their modes of action would be expected to differ from that of the related analogs, 8-azaadenosine, 8-azahypoxanthine, and 8-azainosine, a major feature of whose action is metabolism to phosphates of 8-azaguanosine [5]. We have reported earlier [1] the cytotoxicities of a series of 2-azapurines and their activities as substrates for isolated enzymes; we report here the metabolism and some metabolic effects of 2-azaHyp and 2-azaAdo.

* This work was supported by Grant CH-2W from the American Cancer Society.

† Address all correspondence to: Dr. L. Lee Bennett, Jr., Biochemistry Research, Southern Research Institute, 2000 Ninth Avenue South, P.O. Box 55305, Birmingham, AL 35255.

‡ Abbreviations: 2-azaHyp, 2-azahypoxanthine; 2-azaIno, 2-azainosine; 2-azaAdo, 2-azaadenosine; DTIC, 5 - (3,3 - dimethyl - 1 - triazenyl) - 1*H* - imidazole - 4 - carboxamide; AIC, 4(5)-amino-5(4)-imidazolecarboxamide; H(G)PRT, hypoxanthine (guanine) phosphoribosyltransferase; APRT, adenine phosphoribosyltransferase; HPLC, high pressure liquid chromatography; and TCA, trichloroacetic acid.

MATERIALS AND METHODS

[8-^{14}*C*]-2-*AzaHyp*

This compound was prepared from [8-^{14}C]hypoxanthine in two steps: (a) opening of the purine ring to give [2-^{14}C]AIC; and (b) treatment of the latter compound with nitrite; the procedures are modifications of published methods [6, 7]. The details of the synthesis are given below.

A solution of [8-^{14}C]hypoxanthine (3.21 mg, 1.0 mCi) in 0.4 N NaOH (0.1 ml) was heated in a sealed tube at 150° for 4 hr. The tube was opened, and the contents were streaked across two 8-in. thin-layer plates containing Silica gel GF, 250 μm in thickness (Analtech, Newark, DE). The plates were developed in isopropyl alcohol–NH_4OH–H_2O (8:1:1, by vol.), after which the band containing the product (R_f 0.5) was eluted with hot methanol. Evaporation of the methanol yielded a solid ([2-^{14}C] AIC) which was dissolved in 100 μl of 0.1 N HCl. The solution was added dropwise, using a stream of

nitrogen, to a cold solution of $NaNO_2$ (5 mg) in 50 μl of water. The resulting solution was sequentially (a) refrigerated for 17 hr after which it was diluted with 2 drops of conc. NH_4OH; (b) left 24 hr at ambient temperature; diluted with 10 mg of non-radioactive 2-azaHyp and heated to effect solution, and (c) chilled for several hours. The crystals which formed were collected by filtration (yield 8 mg). The product was purified by thin-layer chromatography on Brinkmann (E. Merck, Darmstadt, West Germany) PLC plates precoated with Silica gel 60F-254, 2 mm thickness, with 1-butanol–H_2O (86:14, v/v) as solvent. The product, obtained by extraction with hot methanol, weighed 5.99 mg and had a specific activity of 1.86 mCi/mmole. A second crop was obtained by adding 10 mg of non-radioactive 2-azaHyp to the mother liquor and purifying as above: yield 6.88 mg, specific activity 0.56 mCi/mmole. The final products were checked for radiopurity by high pressure liquid chromatography (HPLC) on a reverse phase column (see below). The product eluted as a single peak with the retention time (8 min) of known 2-azaHyp; the two likely radioimpurities, [8-^{14}C]hypoxanthine and [2-^{14}C]AIC, were not detected.

2-AzaIMP

This nucleoside was synthesized from 2-azainosine (2-azaIno) by use of nucleoside phosphotransferase (EC 2.7.1.77) prepared from carrots as described by Brunngraber [8]. The procedure has been described in detail elsewhere [9] for preparation of other nucleotides. The final product was characterized by HPLC on a Partisil-10 SAX column and was shown to have a retention time (6 min) about that of IMP and to be of a high degree of purity.

Other materials

2-Azaadenosine was synthesized in our laboratories [10]. New England Nuclear (Boston, MA) was the source of [^{14}C]formate (6.5 mCi/mmole), [8-^{14}C]adenine (5.1 mCi/mmole), and [*methyl*-^{3}H]thymidine (6.7 Ci/mmole). [5-^{3}H]Uridine (10 Ci/mmole) and [8-^{14}C]hypoxanthine (56 mCi/mmole) were obtained from Moravek Biochemicals (Brea, CA) and [4,5-^{3}H]leucine (6 Ci/mmole) was from Schwarz-Mann, Spring Valley, NY. Alkaline phosphatase (17 units/mg) was purchased from the Sigma Chemical Co., St. Louis, MO. 2′-Deoxycoformycin was a gift from Warner-Lambert/Parke Davis Pharmaceutical Research Division, Ann Arbor, MI.

HPLC

HPLC analyses were performed at ambient temperatures with a Waters Associates (Milford, MA) model 202 apparatus. For determination of nucleotides, a Partisil-10 SAX anion exchange column (Whatman, Inc., Clifton, NJ) was used; elution was accomplished with a 40-min linear gradient from 5 mM $NH_4H_2PO_4$, pH 2.8, to 750 mM $NH_4H_2PO_4$, pH 3.7, at a flow rate of 2 ml/min. For analysis of nucleosides and free bases, a reverse phase Partisil ODS-3 column (4.6 × 250 mm) (Whatman, Inc.) was used; elution was accomplished isocratically at a flow rate of 1 ml/min with H_2O–acetonitrile (97:3, v/v). Integration of peak areas was accomplished with a Hewlett–Packard model 3380-A digital electronic integrator. A Perkin–Elmer LC-75 spectrophotometer with autocontrol was used for stop-flow u.v. scanning in confirmation of peak identification. For identification of peaks, the criteria were those described by Brown [11].

Cell cultures

Cultures of HEp-2 cells were grown in SRI-14 medium [12] and cultures of L1210 cells in Fischer's medium [13]. Conditions for each metabolic study are given in the tables and figures.

Study of incorporation of [8-^{14}C]-2-azaHyp into polynucleotides

To determine if ^{14}C from [8-^{14}C]-2-azaHyp was incorporated into polynucleotides, cells were grown in the presence of [8-^{14}C]-2-azaHyp and then fractionated into soluble nucleotides, ribosomal RNA, soluble RNA, "interfacial" RNA, and DNA by the procedures of Caldwell and Henderson [14], which involve the extraction of soluble nucleotides with $HClO_4$ and the isolation of RNA and DNA fractions by extraction with phenol. In some experiments, soluble nucleotides were extracted with boiling 80% ethanol, and polynucleotides were then extracted from the residue with hot 10% NaCl solution from which the polynucleotides were then precipitated by the addition of ethanol [15]. All fractions were assayed for ^{14}C by liquid scintillation spectrometry.

Metabolism of 2-azapurines and their effects on nucleotide pools

For study of the conversion of 2-azapurines to nucleotides and of effects on pools of natural nucleotides, HEp-2 or L1210 cells were grown in the presence of 2-azaHyp or 2-azaAdo for various lengths of time, after which the cells were harvested, washed, and extracted with cold 0.5 N $HClO_4$. After neutralization of the extract with $KHCO_3$ and removal of $KClO_4$, the solution was lyophilized; the residue was dissolved in H_2O, and portions of the solution were subjected to HPLC analysis on a Partisil-10 SAX column. In all experiments in which 2-azaAdo was used, 2′-deoxycoformycin, a known inhibitor of adenosine deaminase [16], was present. In experiments in which a radioactive precursor of purine or pyrimidine nucleotides was used, 2-ml fractions were collected and assayed by liquid scintillation spectrometry.

Effects on synthesis of macromolecules

Effects on the synthesis of DNA, RNA, and protein were determined by measurement of the incorporation of ^{3}H-labeled thymidine, uridine, and leucine by suspension cultures of cells. Cells were incubated for periods of 1–6 hr with [^{3}H-*methyl*] thymidine, [5-^{3}H]uridine, or [4,5-^{3}H]leucine in the absence of inhibitor (controls) or in the presence of the inhibitor over a range of concentrations. When the incubation was complete, cells were harvested, and TCA-insoluble and alkali-stable acid-insoluble fractions were isolated and assayed for radioactivity. Incorporation into TCA-insoluble material is a measure of protein synthesis when leucine is the precursor, of DNA synthesis when thymidine is the precursor, and of synthesis of RNA and DNA when uridine

is the precursor. Radioactivity in the alkali-stable fraction is a measure of DNA synthesis from thymidine or from [5-^{3}H] uridine. Radioactivity in RNA was calculated as the difference between activities in the TCA-insoluble and the alkali-stable fractions. The same procedure was used in experiments with other radioactive precursors ([^{14}C]formate or [8-^{14}C] adenine). These methods are modifications of those of Hershko *et al.* [17] and have been described in detail elsewhere [18].

Phosphoribosyltransferase activities

The activities of [8-^{14}C]-2-azaHyp, [8-^{14}C]adenine and [8-^{14}C]hypoxanthine for phosphoribosyltransferases were determined as described elsewhere [9]. The preparation was a 100,000 *g* supernatant fraction from HEp-2 cells that had been dialyzed overnight against 0.05 M Tris. The incubation mixture contained, in a final volume of 0.20 ml, a ^{14}C-labeled base, 5′-phosphoribosyl 1-pyrophosphate (PRPP) (0.8 μmole), $MgCl_2$ (0.40 μmole), and Tris buffer, pH 7.6. The nucleotide formed was isolated by paper chromatography and assayed for ^{14}C in a liquid scintillation spectrometer.

IMP dehydrogenase

IMP dehydrogenase studies were performed with enzyme from L1210 cells prepared as described elsewhere [19]. Enzyme activity was determined spectrophotometrically by measurement of change in absorbance at 340 nm as NAD was reduced [20].

RESULTS

2-AzaHyp as a substrate for hypoxanthine (guanine) phosphoribosyltransferase [H(G)PRT](EC 2.4.2.8)

To identify the phosphoribosyltransferase responsible for nucleotide formation from 2-azaHyp, studies were performed with supernatant fractions from HEp-2 cells that had activity of both H(G)PRT and adenine phosphoribosyltransferase (APRT). Under these conditions nucleotide formation from 2-azaHyp, adenine, and hypoxanthine was linear for at least 30 min. As shown in Fig. 1, the rate of conversion of 2-azaHyp to nucleotide was considerably slower than that for adenine or hypoxanthine. The conversion of 2-azaHyp to nucleotide was inhibited by more than 50% by an equimolar amount of hypoxanthine, whereas this concentration of adenine did not affect the conversion. Conversely, 2-azaHyp inhibited the conversion of hypoxanthine to IMP, whereas it was ineffective, at a concentration 20-fold that of adenine, in reducing the conversion of adenine to AMP. Thus, 2-azaHyp appears to be a substrate for H(G)PRT and not for APRT.

Examination of the possible incorporation of metabolites of 2-azaHyp into polynucleotides

Experiments were performed to determine if there was detectable incorporation of ^{14}C in the polynucleotides of HEp-2 cells grown in the presence of [8-^{14}C]-2-azaHyp. Cells in suspension culture (approx. 4×10^5 cells/ml) were grown in the presence of [8-^{14}C]-2-azaHyp (6 nCi/ml) for periods of 2–

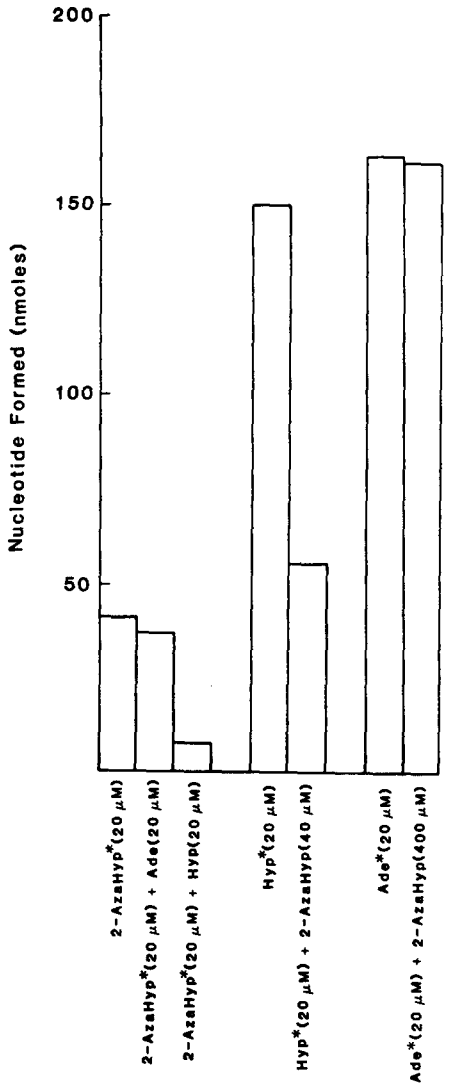

Fig. 1. Competition between 2-azaHyp, adenine, and hypoxanthine for phosphoribosyltransferases in enzyme preparations from HEp-2 cells. The incubation mixture contained the enzyme preparation, PRPP, $MgCl_2$ and radioactive and nonradioactive bases at the concentrations indicated in the figure. Labeled substrates ([8-^{14}C]2-azaHyp, [8-^{14}C]Hyp, or [8-^{14}C]Ade) are indicated by asterisks. The values shown for nucleotide formation from the radioactive substrate are the total formed during an incubation period of 30 min. See text for details.

24 hr. In some experiments isolation of polynucleotides was accomplished by the procedure of Caldwell and Henderson [14] in which cells are worked up by phenol extraction to yield soluble nucleotides, soluble RNA, ribosomal RNA, "interfacial" RNA and DNA. The soluble nucleotide fraction had significant radioactivity, but none of the polynucleotide fractions contained detectable amounts of ^{14}C. In other experiments, soluble nucleotides were removed by extraction with cold 0.5 N $HClO_4$ and combined RNA and DNA were extracted with hot 10% NaCl solution. The sodium nucleates, precipitated by the addition of ethanol,

contained detectable amounts of ^{14}C. When the sodium nucleates were subjected to chromatography on an anion exchange column, essentially all of the ^{14}C present eluted in the breakthrough peak and therefore represents a non-polar substance or substances, which is not retained on the column. When the supernatant fractions from the precipitation of the sodium nucleates were examined by anion-exchange chromatography, ^{14}C was present in the breakthrough peak and, when examined by reverse phase chromatography, was present at the retention time of 2-azaHyp. The ^{14}C in the sodium nucleates, therefore, appears to be due to contamination with 2-azaHyp. Thus, all of these experiments failed to detect any incorporation of ^{14}C from 2-azaHyp into polynucleotides. Since these experiments were all negative the results are not shown.

Conversion of 2-azaHyp and 2-azaAdo to soluble nucleotides

Nucleotide formation in intact HEp-2 cells was studied with both ^{14}C-labeled and non-radioactive 2-azaHyp and with non-radioactive 2-azaAdo. The first experiments were performed with [8-^{14}C]-2-azaHyp and were designed to examine the formation of both ribonucleotides and deoxyribonucleotides. Later experiments were performed with unlabeled compounds. These two types of experiments are discussed separately.

(A) *Experiments with* [8-^{14}C]-2-*azaHyp.* To suspension cultures of HEp-2 cells in 500 ml of medium, [8-^{14}C]-2-azaHyp was added at a concentration of 4 nCi (0.9 μg)/ml. After 4 hr the cells were harvested by centrifugation, washed free of medium with 0.85% NaCl solution, and finally extracted with cold 0.5 N $HClO_4$. The $HClO_4$ extract was treated with $KHCO_3$, and the resulting precipitate of $KClO_4$ was removed by centrifugation. A portion of the extract was subjected to HPLC analysis on a Partisil-10 SAX ion exchange column, and 2-ml fractions were collected. As shown in Fig. 2, radioactivity was found in the breakthrough peak and in peaks with retention times of 7–8, 17–19, and 34–36 min. These retention times are slightly greater than those for AMP, ADP, and ATP. The relative amounts of ^{14}C present in each area were: breakthrough, 6%; monophosphate area, 21%; diphosphate area, 28%; triphosphate area, 45%. Another portion of the $HClO_4$ extract was treated with excess alkaline phosphatase, and the mixture was then subjected to chromatography on a reverse phase column with H_2O-acetonitrile as the eluant (see above). Radioactivity was found in a single peak with a retention time of 18 min, the same as that of 2-azaAdo, which is well separated from 2-azaIno (9 min). These results thus indicate that the principal metabolites of 2-azaHyp are nucleotides of 2-azaadenine. Additional studies of nucleotide formation from both 2-azaHyp and 2-azaAdo were accomplished with non-radioactive substrates as described below in Section B.

Another portion of the initial cell extract was separated into ribonucleotide and deoxynucleotide fractions by chromatography on a boronate column [Affi-Gel-601 (BioRad, Richmond, CA)]. This was accomplished by passage of the entire remainder of the fraction through a column (1 × 30 cm) of Affi-Gel-601 using a 0.05 M glycine buffer containing 0.025 M magnesium acetate, pH 8.5, for loading and as the eluant. The effluent deoxynucleotide fraction, i.e. that not retained on the column, was divided into two parts. The smaller part was subjected to HPLC analysis on a Partisil-10 SAX column, and fractions were collected. ^{14}C was found only in the breakthrough peak. This finding indicates that ribonucleotides had been retained, as expected, on the column, and that any ^{14}C present in deoxynucleotides was below the limit of detection in the sample assayed. The other part of the fraction not retained

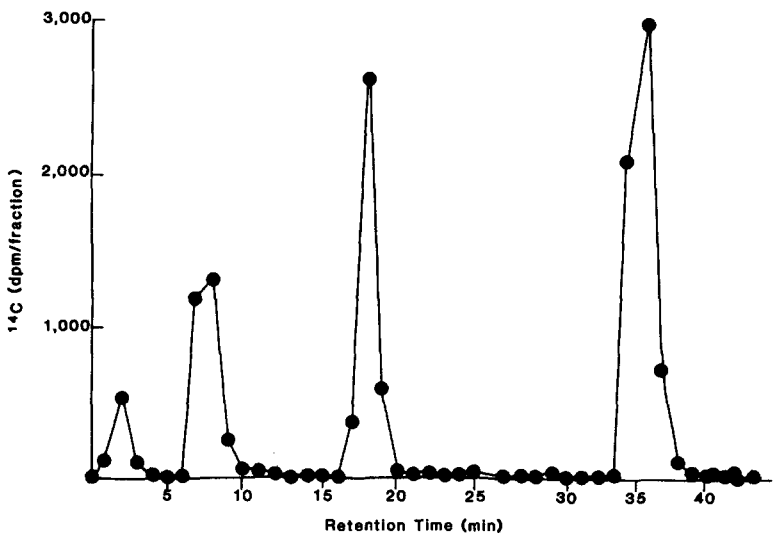

Fig. 2. Analysis by HPLC of the radioactivity in the soluble fraction of HEp-2 cells grown in the presence of [8-^{14}C]-2-azaHyp. Cells were grown in the presence of [8-^{14}C]-2-azaHyp [4 nCi (0.9 μg)/ml] for 4 hr, after which the cells were harvested and extracted with cold 0.5 N $HClO_4$. This extract was subjected to HPLC on a Partisil-10 SAX anion exchange column. Fractions of 2 ml each were collected and assayed for radioactivity in a liquid scintillation spectrometer. See text for details.

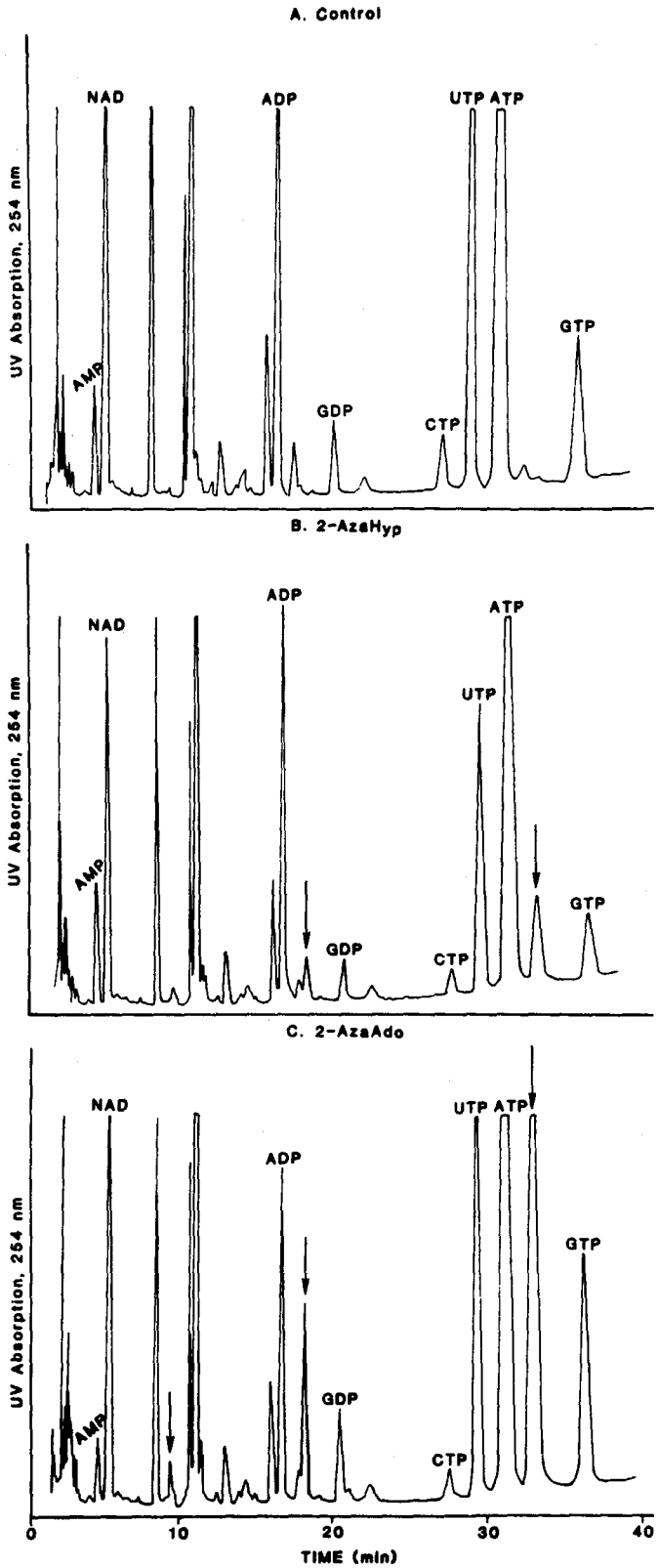

Fig. 3. Metabolism of 2-azaHyp and 2-azaAdo. To HEp-2 cells in suspension culture (10^6 cells/ml) were added 2-azaHyp (final concentration 74 μM) or 2-azaAdo (final concentration 19 μM). After 4 hr the cells were harvested and extracted with cold 0.5 N $HClO_4$. This extract was subjected to HPLC on a Partisil-10 SAX anion exchange column as described in Fig. 2 and in the text. In the experiments with 2-azaAdo, deoxycoformycin was present at a concentration of 0.35 μM. The arrows mark the new peaks appearing in the cells treated with 2-azaHyp and 2-azaAdo.

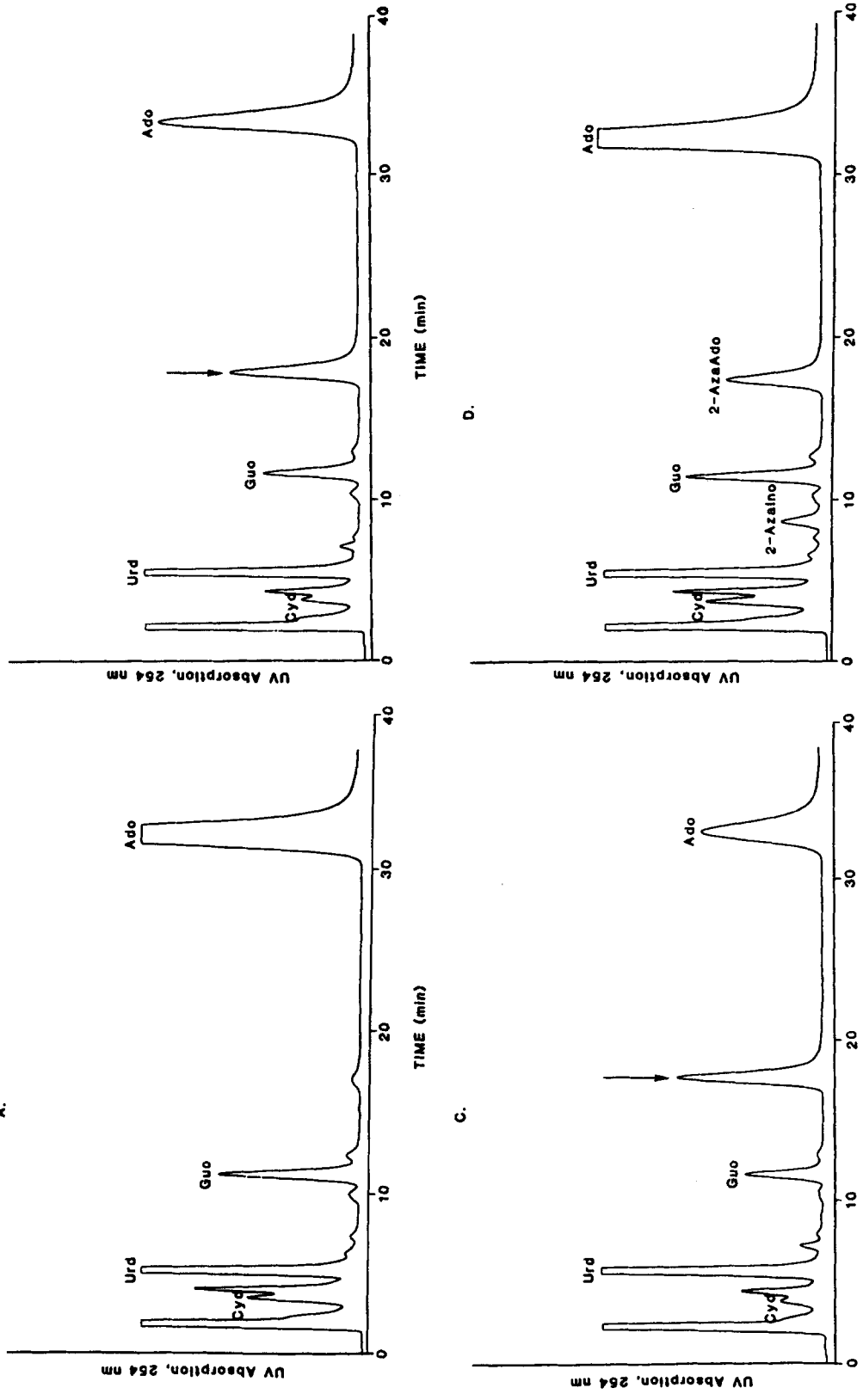

Fig. 4. Identification of the metabolites of 2-AzaHyp and 2-azaAdo. Extracts from control cells and from cells that had been grown for 24 hr in the presence of 2-azaHyp (74 μM) or 2-azaAdo (19 μM) (see Table 1 and Fig. 3) were treated overnight with alkaline phosphatase. The extract was then subjected to reverse phase chromatography as described in the text. In the experiment with 2-azaAdo, deoxycoformycin was present at a concentration of 0.35 μM. Key: (A) control cells; (B) cells treated with 2-azaHyp; and (C) cells treated with 2-azaAdo. Panel D is from the same cell extract as panel A except that before chromatography there were added 10 nmoles each of 2-azaHyp, 2-azaAdo, and 2-azaIno. Arrows mark new peaks appearing in the cells treated with 2-azaHyp or 2-azaAdo.

on Affi-Gel was concentrated by lyophilization and treated overnight with alkaline phosphatase. The incubation mixture was immersed in a boiling water bath and then centrifuged. The supernatant solution was lyophilized to dryness and taken up in 0.5 ml of H_2O. Portions of this solution were chromatographed on a reverse phase column. Radioactivity was found in the breakthrough peak, which would contain 2-azaHyp, but none was present in fractions corresponding to 2-azadAdo. Thus, any conversion of [^{14}C]-2-azaHyp to deoxynucleotides of 2-azaadenine was below the limit of detection in these experiments.

As an additional confirmatory step, the Affi-Gel column was eluted with 0.025 M ammonium acetate buffer, pH 5, to remove ribonucleotides, which should have been retained. This eluate, after appropriate concentration, was chromatographed on a Partisil-10 SAX column. Most of the ^{14}C was present in peaks with retention times about the same as those of ATP and ADP, thus confirming, as had already been shown with the initial cell extract, that 2-azaHyp was converted to phosphates of 2-azaAdo.

(B) *Experiments with non-radioactive 2-azaHyp and 2-azaAdo.* HPLC analyses were made of extracts of cells grown in the presence of 2-azaHyp or 2-azaAdo. In each of these extracts, a new diphosphate and a new triphosphate were present, each retained longer than ADP or ATP. Typical HPLC profiles are shown in Fig. 3 (B and C). To confirm the identity of these peaks, several types of evidence were obtained. First, the peaks were scanned as they were eluted from the ion exchange column. The scan showed absorption maxima at 259 and 300 nm; this spectrum was indistinguishable from that of known 2-azaAdo in the same buffer. Another aliquot of the cell extract was treated with alkaline phosphatase and then chromatographed on a reverse phase column, with the results shown in Fig. 4 (A, B, C). In addition to the natural nucleosides, a nucleoside with a retention time of 18–19 min was obtained; this is the same retention time as that of known 2-azaAdo. This peak was scanned and had an ultraviolet absorption spectrum indistinguishable from that of a known sample of 2-azaAdo placed on the column and eluted under the same conditions. Additional evidence that the peak was 2-azaAdo was obtained by a "reinforcement" experiment; 2-azaAdo, added to the sample before chromatography, specifically augmented the size of this peak (result not shown). Figure 4D shows the chromatography of another sample of the extract used for Fig. 4A except that before chromatography known samples of 2-azaHyp, 2-azaAdo, and 2-azaIno were added to the extract. The new peaks resulting from this treatment were at approximately 9 min, which is the retention time of 2-azaIno, as determined in a separate experiment, and one at about 18 min, corresponding to 2-azaAdo; 2-azaHyp was not resolved under these conditions. The absence of a significant peak at 9 min in panels B and C indicates that at best only very small amounts of 2-azaIno or its phosphates were present in the cells grown in the presence of 2-azaHyp or 2-azaAdo.

Other experiments were performed to compare the extent of metabolism of 2-azaHyp and 2-azaAdo to nucleotides. As shown in Table 1, 2-azaAdo was converted to 2-azaATP much more effectively than was 2-azaHyp. At periods up to 4 hr 4- to 5-fold more 2-azaATP was formed from 19 μM 2-azaAdo than was formed from 74 μM 2-azaHyp. However, at 24 hr the ratio had decreased to 2-fold or less.

Effects on nucleotide pools

2-AzaHyp, after short periods of exposure, selectively reduced the pools of guanine nucleotides; after 24 hr, the pools of guanine nucleotides had recovered (Table 2). In the presence of 2-azaAdo the pools of adenine nucleotides were selectively reduced at short times, but at 24 hr the pools of all nucleotides were reduced. In Table 2 all of the individual experiments are shown to illustrate the nature of the data obtained. Considerable variations were noted in effects on nucleotide pools, particularly with the pyrimidine nucleotides. The variability in effects on cytidine nucleotide pools probably resulted from the small size of these pools and the consequent difficulty in their measurement. The variations in the duplicate

Table 1. Conversion of 2-azaHyp and 2-azaAdo to 2-azaATP in HEp-2 cells*

	2-AzaATP (nmoles/10^9 cells)		
	1 hr	4 hr	24 hr
Experiment 1			
2-AzaHyp (74 μM)		1,853	1,927
2-AzaAdo (19 μM) + deoxycoformycin (0.35 μM)		10,354	2,390
Experiment 2			
2-AzaHyp (74 μM)		2,904	
Experiment 3			
2-AzaAdo (19 μM) + deoxycoformycin (0.35 μM)	15,080	18,719	1,134

* HEp-2 cells were grown in the presence of 2-azaHyp or 2-azaAdo for the indicated times. The cells were extracted, and the extract was subjected to HPLC on an anion exchange column (see text). In experiment 1, 2-azaHyp and 2-azaAdo were assayed in parallel with cells out of the same batch. Experiments 2 and 3 were performed independently with each agent. The results of all three experiments are given to illustrate the range of values obtained.

Table 2. Effects of 2-azaHyp and 2-azaAdo on pools of natural nucleotides of HEp-2 cells*

	Time (hr)	Nucleotides (% control)			
		Adenine	Guanine	Uracil	Cytosine
2-AzaHyp (74 μM)	4	71	62	77	80
	4	93	78	115	103
	4	76	42	79	56
	4	78	56	67	48
	4	84	55	109	85
	24	45	85	56	88
	24	76	101	120	131
2-AzaAdo (19 μM) + deoxycoformycin (0.35 μM)	4	81	153	84	48
	4	26	72	84	82
	4	27	93	85	81
	24	31	61	37	109
	24	15	40	37	20

* Cells were grown for the indicated times in the presence of either 2-azaHyp or 2-azaAdo, and the nucleotide pools were determined by HPLC. Untreated cells were used as controls for each time; each pool is shown in the table as a percent of that of the control culture for that time. Pool sizes were calculated on the basis of cell counts made at the beginning of the experiments. Some of the experiments are the same as those from which the data of Table 1 were drawn. See text for details.

24-hr experiments may be due to the fact that by this time some cells probably had begun to lyse. There is no obvious explanation for the fact that in the first of the 4-hr experiments with 2-azaAdo adenine nucleotides were only slightly reduced whereas in the other two experiments they were reduced by about 75%.

Effects on incorporations of precursors into soluble nucleotides

2-AzaAdo at a concentration of 3.7 μM produced a modest inhibition of the incorporation of [^{14}C] formate into purine nucleotides. 2-AzaHyp at a consideraly higher, but approximately equitoxic concentration, produced a greater inhibition (Fig. 5). Neither agent had significant effects on the conversion of [2-^{14}C]uridine or [2-^{14}C]thymidine to nucleotides as determined in experiments similar to those with formate (results not shown).

Effects on synthesis of macromolecules (Fig. 6)

2-AzaHyp at a concentration of 74 μM had no effect on the utilization of leucine or uridine but did produce a modest inhibition of utilization of thymidine. This selective effect on thymidine utilization was observed consistently in several experiments. 2-AzaAdo at a concentration of 1.9 μM inhibited the incorporation of all three precursors to about the same extent. Both 2-azaHyp and 2-azaAdo were assayed over a broad range of concentrations both above and below those shown in the figure. 2-AzaHyp at a concentration of 110 μM was no more effective than it was at a concentration of 74 μM. 2-AzaAdo at a concentration of 0.4 μM was without effect on utilization of any of the precursors. The results shown in Fig. 6 are for the TCA-insoluble fractions; in all experiments, alkali-stable (DNA) fractions were also assayed and the radioactivity in DNA and RNA was calculated. Since these determinations revealed no further selective action, the results are not shown. Both 2-azaHyp and 2-azaAdo were also assayed by the same procedure for effects on the utilization of other labeled precursors: adenine, formate, and hypoxanthine. The results (not shown) revealed no specific effects.

Inhibition of IMP dehydrogenase (Fig. 7)

2-AzaIMP inhibited IMP dehydrogenase and the inhibition was competitive with IMP. The apparent K_i was 66 μM; the apparent K_m for IMP was 10 μM.

Reversal studies

A number of attempts were made to reverse the toxicities of 2-azaHyp or 2-azaAdo to HEp-2 cells by addition to the medium of various candidate reversal agents. We have used this method for many other inhibitors (see, for example, Ref. 9). A concentration of inhibitor was used that gave 65–95% inhibition of colony formation; the candidate agents were added at the same time as the inhibitor and the colonies formed were counted 10 days later. These results are not shown because of their lack of reproducibility. Thus, when 2-azaHyp was the inhibitor, a significant degree of reversal or prevention of inhibition was obtained in three of six experiments with adenine, one out of five experiments with guanine, and four out of five experiments with adenine + guanine. The only clear-cut observation was that the toxicity of 2-azaAdo (in the presence of deoxycoformycin) was not reversed by any of the agents or combinations assayed: adenine, AIC, guanine, uridine, adenine + guanine, adenine + uridine, or adenine + uridine + guanine.

DISCUSSION

Certain 2-azapurines have been known for many years to be biologically active (for references to older

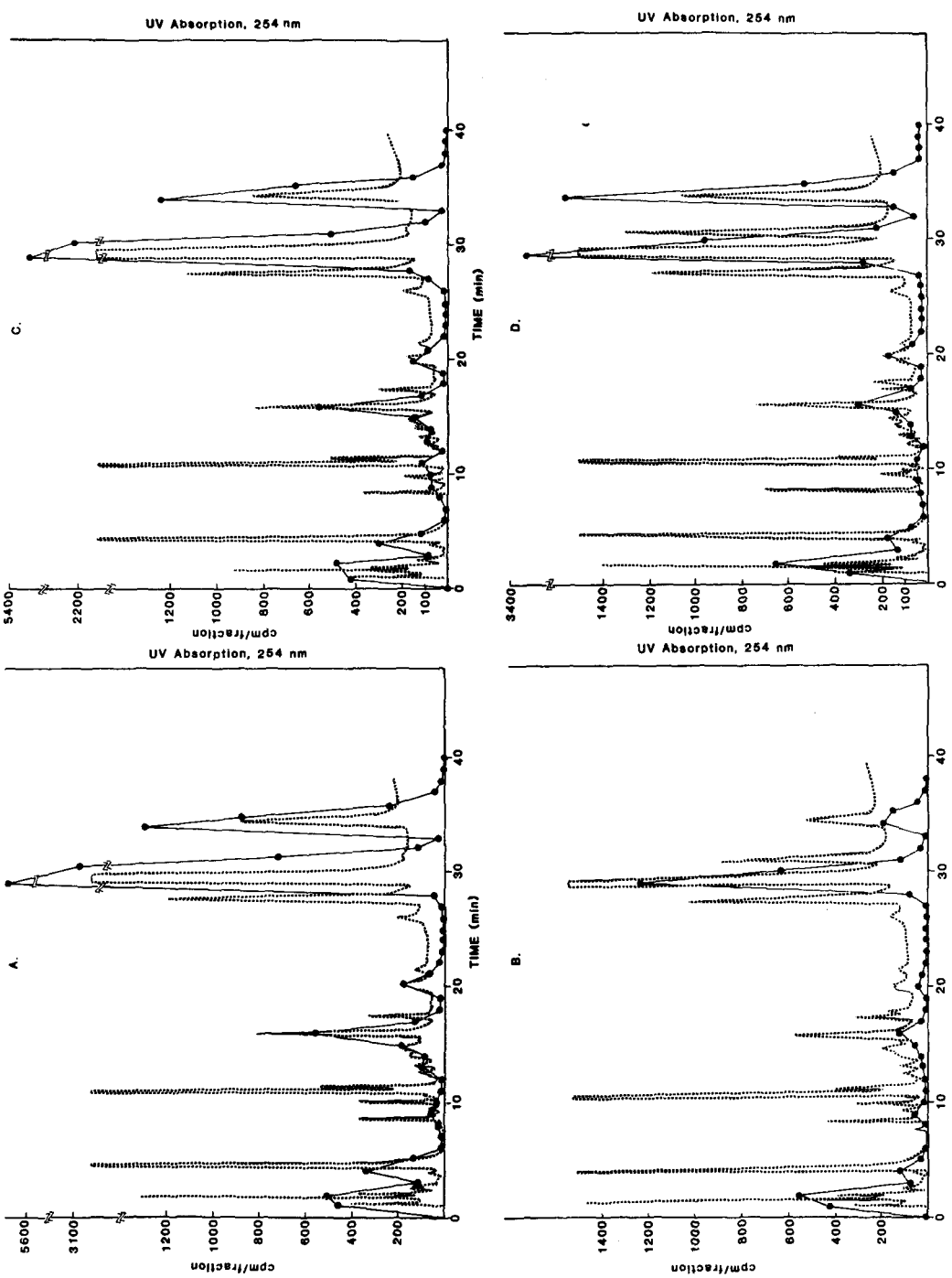

Fig. 5. Effects of 2-azaHyp and 2-azaAdo on the incorporation of [^{14}C]formate into nucleotides. HEp-2 cells were grown in suspension cultures as described in Fig. 3. To these cultures were added 2-azaHyp (final concentration 74 μM) or 2-azaAdo (final concentration 3.7 μM), followed 0.5 hr later by [^{14}C]formate (0.05 μCi/ml). Control cultures received only [^{14}C]formate. In the experiment with 2-azaAdo, deoxycoformycin was present at a concentration of 0.35 μM. Four hours after addition of [^{14}C]formate the cells were harvested, and an $HClO_4$ extract was prepared and subjected to HPLC on a Partisil-10 SAX column as described in the text. Fractions of 2 ml were taken and assayed for radioactivity by liquid scintillation spectrometry. Solid lines: radioactivity; broken lines: u.v. absorption. Key: (A) control; (B) 2-azaHyp; (C) control; and (D) 2-azaAdo. Separate controls are shown for the experiment with 2-azaHyp (A) and for the experiment with 2-azaAdo (C).

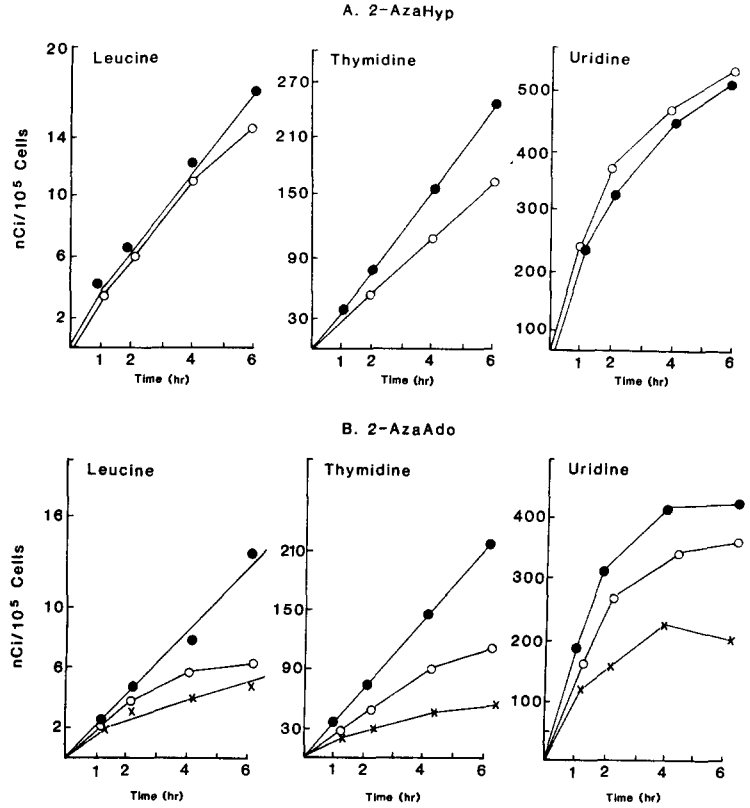

Fig. 6. Effects of 2-azaHyp and 2-azaAdo on macromolecular synthesis. To HEp-2 cells (~3 × 10^5 cells/ml) were added 2-azaHyp (final concentration 74 μM) or 2-azaAdo (final concentration 9.5 or 1.9 μM), followed 0.5 hr later by [4,5-^{3}H]L-leucine, [^{3}H-*methyl*]thymidine or [5-^{3}H]uridine, each at a final concentration of 2 μCi/ml. In the experiment with 2-azaAdo, deoxycoformycin was present at a concentration of 0.35 μM. Control cultures received only one of the radioactive substrates. Samples of each culture were taken at 1, 2, 4, and 6 hr after administration of the labeled compound, and extracted with cold TCA. The TCA-insoluble fractions were assayed for radioactivity by liquid scintillation spectrometry. Key: (A) (●—●) control, and (○—○) 2-azaHyp (74 μM); (B) (●—●) control, (○—○) 2-azaAdo (1.9 μM), and (×—×) 2-azaAdo (9.5 μM). The abscissas are the times after the administration of the labeled substrates at which cells were harvested.

literature, see Ref. 1) but little is known about their mode(s) of action. We have reported a study in cell culture with a series of 2-azapurines and their nucleosides, which showed that 2-azaAdo was the most toxic of the compounds studied and that, like most other purine analogs, the 2-azapurines were toxic only if the cells had the capacity to convert them to nucleotides [1]. The present study was initiated as an investigation of the mode of action of 2-azaHyp, taken as a representative of this class of agent; when it was found that the principal metabolite of 2-azaHyp was 2-azaATP, 2-azaAdo was included in the study as a related toxic compound that would also be a source of 2-azaATP.

As would be expected fom its structure and from the observation that cells lacking H(G)PRT were resistant to it [1, 4], 2-azaHyp was a substrate for H(G)PRT but not for APRT (Fig. 1). That it is a good substrate is shown by the fact that it competed well with hypoxanthine. The fact that 2-azaATP was a metabolite of 2-azaHyp indicates that 2-azaIMP is metabolized via the same pathway by which IMP is converted to adenine nucleotides. The fact that no 2-azaIMP was detected in cells grown in the presence of 2-azaHyp probably is explained by the efficient conversion of 2-azaIMP, as it is formed, to adenine nucleotides.

Since the phosphates of 2-azaAdo were the only observed metabolites of 2-azaHyp, and since no conversion to deoxynucleotides or incorporation into polynucleotides was detected, it is likely that the ribonucleotides of 2-azaadenine, particularly 2-azaATP, are responsible for the biological activity of 2-azaHyp. However, incorporation of very small amounts of 2-azaHyp (as 2-azaadenine) into polynucleotides, or the formation of small amounts of 2-aza-2′-deoxynucleotides, cannot be completely excluded; such conversions, if they occurred, were not detectable by the methods employed. The incorporation of 2-azaAdo was not examined because labeled 2-azaAdo was not available. It is conceivable that the larger pools of 2-azaATP from 2-azaAdo might have resulted in detectable incorporation into polynucleotides whereas the smaller pools derived from 2-azaHyp would not. However, even the pool of 2-azaATP produced by 2-azaHyp is considerable,

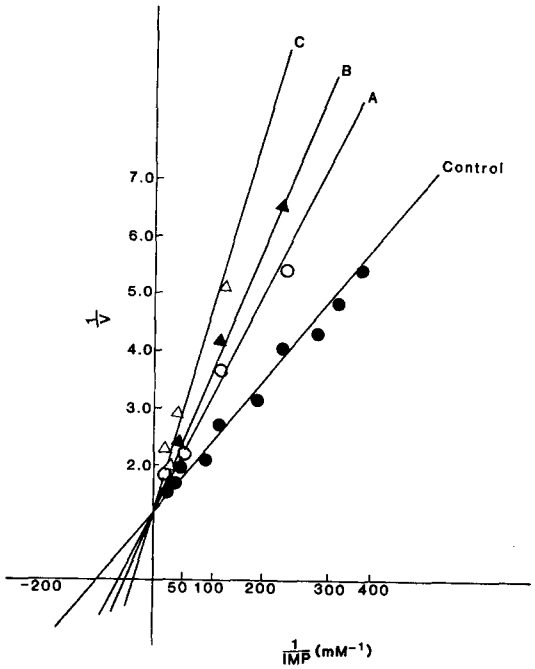

Fig. 7. Inhibition of IMP dehydrogenase by 2-azaIMP. The incubation mixture contained IMP, 50 μM; NADH, 200 μM; the enzyme (28 μg protein), and either no further additions (control) or 2-azaIMP at the following μM concentrations: (A) 39; (B) 68; and (C) 140. The reaction was followed spectrophotometrically (see reference in text). Ordinate, reciprocals of velocity in nmoles/min.

and overall the results would indicate that any incorporation of 2-azaAdo could be at best very low. These results are in contrast to those with 8-azaadenosine which is incorporated extensively into polynucleotides [5].

Since 2-azaATP was the principal metabolite of both 2-azaHyp and 2-azaAdo, it would be likely that these compounds have the same mechanism of action. Consistent with this concept are the facts that, in comparison with 2-azaHyp, 2-azaAdo was a much better source of 2-azaATP and was also much more cytotoxic. However, the results show some differences in metabolic effects of these two agents. Thus, (a) 2-azaHyp consistently produced a modest selective effect on DNA synthesis, as determined by thymidine incorporation, whereas 2-azaAdo had no selective effect on synthesis of macromolecules (Fig. 6); (b) 2-azaHyp selectively reduced pools of guanine nucleotides, whereas 2-azaAdo did not (Table 2); and (c) purines had no effects in reversing or preventing inhibition of cell growth by 2-azaAdo, whereas they produced some reversal or prevention, albeit highly variable, of inhibition by 2-azaHyp. Both agents inhibited the incorporation of formate into purine nucleotides (Fig. 5). This latter observation indicates an inhibition on the purine biosynthetic pathway; however, the failure of purines to give consistent reversal of cytotoxicity would indicate that this blockade is not the primary site of action of either agent. Saunders and Chao [4] have reported that toxicity of 2-azaHyp to Chinese hamster ovary cells may be prevented by the presence of hypoxanthine, adenine, guanine, or AIC-ribonucleoside; we have no rationalization for the difference in consistency of response in these experiments and in ours with HEp-2 cells. These same workers have reported the inhibition by 2-azaHyp of the utilization of formate, uridine, and thymidine.

The difference in action of 2-azaHyp and 2-azaAdo possibly may be rationalized. 2-AzaIMP is an obligate metabolite in the conversion of 2-azaHyp to 2-azaATP. However, since 2-azaAdo was always used in combination with deoxycoformycin, 2-azaIMP would not be expected as a metabolite of this nucleoside. The observed selective decrease of guanine nucleotide pools produced by 2-azaHyp, but not by 2-azaAdo, might thus be an effect caused by 2-azaIMP. Finally, it is known that agents that reduce the pools of GTP inhibit DNA synthesis [21], and that selective inhibition of DNA synthesis by 2-azaHyp, but not by 2-azaAdo, was, in fact, observed. This line of reasoning led to the assay of 2-azaIMP as an inhibitor of IMP dehydrogenase (Fig. 7). 2-AzaIMP was, in fact, an inhibitor of this enzyme with a K_i of 66 μM. This nucleoside is thus not a potent inhibitor, and this fact, together with our failure to detect 2-azaIMP in cells treated with 2-azaHyp, would indicate at first glance that inhibition of IMP dehydrogenase might not be expected in cells treated with 2-azaHyp. However, inhibition by 2-azaIMP is competitive with IMP, and the degree of inhibition of the dehydrogenase would be determined by the ratio of 2-azaIMP to IMP. The concentrations of both IMP and 2-azaIMP were below the level of detection in the cells studied, and therefore no estimate can be made of the relative amounts of these nucleotides. However, the facts that 2-azaHyp selectively reduced pools of guanine nucleotides and that 2-azaIMP inhibited the dehydrogenase constitute strong presumptive evidence that the 2-azaIMP/IMP ratio in the cells is favorable to inhibition.

A notable observation is the attainment of large pools of 2-azaATP in cells treated with 2-azaAdo (Table 1). These pools equal or exceed those of ATP in control cells. At the same time the pools of ATP were strongly depressed (Table 2). This is not an action unique to 2-azaAdo, for other adenosine analogs, e.g. 2-fluoroadenosine [22], shows the same phenomenon. The decrease in pools of ATP is sufficiently great that it alone might explain the cytotoxicity of 2-azaAdo. However, it would be expected that the large pool of 2-azaATP would itself produce some specific inhibitions, and the overall effects of 2-azaAdo would be the sum of the effects of reduced ATP and the effects produced by 2-azaATP. The multiplicity of potential sites of action probably is responsible for our failure to detect a blockade that might be considered primary. In addition to the experiments in this study we have performed other more limited studies in a search for sites of action of 2-azaAdo; this agent was without effect on the concentration of adenosylhomocysteine in intact cells and on the synthesis of polyadenylic acid.* Thus, 2-azaAdo at present must be considered as

* L. L. Bennett, Jr., D. Smithers, P. W. Allan and L. White, unpublished results.

another highly toxic adenosine analog whose primary site of action is still undefined.

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