*Proc. Natl. Acad. Sci. USA*
Vol. 79, pp. 5127–5131, September 1982
Biochemistry

# Purine oversecretion in cultured murine lymphoma cells deficient in adenylosuccinate synthetase: Genetic model for inherited hyperuricemia and gout

(inosine/purine salvage/hypoxanthine/guanine phosphoribosyltransferase)

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*Communicated by James B. Wyngaarden, May 11, 1982*

**ABSTRACT** **Alterations in several specific enzymes have been associated with increased rates of purine synthesis *de novo* in human and other mammalian cells. However, these recognized abnormalities in humans account for only a few percent of the clinical cases of hyperuricemia and gout. We have examined in detail the rates of purine production *de novo* and purine excretion by normal and by mutant (AU-100) murine lymphoma T cells (S49) 80% deficient in adenylosuccinate synthetase [IMP:L-aspartate ligase (GDP-forming), EC 6.3.4.4]. The intracellular ATP concentration of the mutant cells is slightly diminished, but their GTP is increased 50% and their IMP, four-fold. Compared to wild-type cells, the AU-100 cells excrete into the culture medium 30- to 50-fold greater amounts of purine metabolites consisting mainly of inosine. Moreover, the AU-100 cell line overproduces total purines. In an AU-100-derived cell line, AU-TG50B, deficient in adenylosuccinate synthetase and hypoxanthine/guanine phosphoribosyltransferase (IMP:pyrophosphate phosphoribosyltransferase, EC 2.4.2.8), purine nucleoside excretion is increased 50- to 100-fold, and *de novo* synthesis is even greater than that for AU-100 cells. The overexcretion of purine metabolites by the AU-100 cells seems to be due to the primary genetic deficiency of adenylosuccinate synthetase, a deficiency that requires the cell to increase intracellular IMP in an attempt to maintain ATP levels. As a consequence of elevated IMP pools, large amounts of inosine are secreted into the culture medium. We propose that a similar primary genetic defect may account for the excessive purine excretion in some patients with dominantly inherited hyperuricemia and gout.**

Hyperuricemia in humans results from either diminished renal clearance or excessive purine production (1). In the latter case, alterations in several specific enzymes have been associated with increased rates of *de novo* purine synthesis (2–9). However, the pathological mechanisms involved in most cases of overproduction hyperuricemia are unknown but are likely due to other alterations in the *de novo* purine synthetic pathway, which is subject to a variety of regulatory mechanisms (10–18).

We have isolated from a population of murine T-lymphoma cells (S49) a mutant clone, AU-100, which is 80% deficient in adenylosuccinate synthetase [IMP:L-aspartate ligase (GDP-forming), EC 6.3.4.4] (19), the penultimate enzyme in the *de novo* biosynthesis of adenosine nucleotides. This cell line is prototrophic for purines and contains increased levels of IMP, GMP, and GTP (19). Levels of hypoxanthine/guanine phosphoribosyltransferase (HGPRTase; IMP:pyrophosphate phosphoribosyltransferase, EC 2.4.2.8) measured in gel-filtered extracts of AU-100 cells are greater than those found in wild-type cell extracts, but the AU-100 cells do not incorporate labeled hypoxanthine from the medium as efficiently as do wild-type cells (19). This latter observation was postulated to result from a physiologically inhibited HGPRTase *in situ* (19). Studies with other cell lines have shown that a deficiency in HGPRTase is associated with increased rates of purine excretion into the medium (20, 21) and increased purine synthesis *de novo* (20, 22–24). Because hypoxanthine uptake from the medium was depressed in the AU-100 cell line (19), we reexamined the rates of *de novo* intracellular purine production and determined the rates of purine metabolite excretion in this adenylosuccinate synthetase-deficient cell line. The AU-100 cells, as compared to wild-type cells, overproduce purines *de novo* by 4-fold, secrete 30- to 50-fold greater quantities of purine nucleoside in the form of inosine into the medium, but generate adenine and guanine nucleotides at near normal rates that reflect their relative steady-state concentrations.

## METHODS

**Cell Culture and Mutant Selection.** The growth and lymphocytic properties of wild-type and mutant S49 cells have been described in detail (25–27). The isolation and characterization of the AU-100 (19) (adenylosuccinate synthetase-deficient) and the MPR3-3 (HGPRTase-deficient) (28) cell lines also have been reported. A spontaneous HGPRTase-deficient derivative of AU-100 cells, AU-TG50B, was cloned and isolated from semisolid agarose containing 50 μM 6-thioguanine.

***De Novo* Purine Synthesis Rates.** To determine the rates of *de novo* purine synthesis, cells were grown for 18 hr in complete medium to assure asynchronous and exponential growth, and then the rates of [U-$^{14}$C]glycine (0.1–0.3 μCi/ml; 1 Ci = 3.7 × $10^{10}$ becquerels) or [$^{14}$C]formate (0.2–0.5 μCi/ml) incorporation into intracellular purines and proteins were measured by the silver precipitation method of Martin and Owen (29) as reported by Ullman *et al.* (19). In order to measure purines excreted into the medium, the cell pellet was removed by centrifugation, and the serum proteins in the culture medium were precipitated in 1 M perchloric acid. The precipitated protein was removed by centrifugation, and the supernatant was boiled for 30 min, followed by addition of concentrated $NH_4OH$ until the pH was >9.0. The purines were isolated by the silver precipitation method after addition of 5 μmol of carrier hypoxanthine and counted as described (29). The incorporation of [U-

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Abbreviations: HGPRTase, hypoxanthine/guanine phosphoribosyltransferase; EHNA, *erythro*-9-[3-(2-hydroxynonyl)]adenine.

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$^{14}$C]glycine and [$^{14}$C]formate into intracellular and extracellular purines and into intracellular proteins, RNA, and DNA was linear with cell densities to $10^6$ cells per ml.

Intracellular glycine pools were measured in perchloric acid-soluble extracts from $10^8$ exponentially growing cells on a Beckman amino acid analyzer and quantitated by reaction with ninhydrin and comparison to standards.

**Identification of Excreted Purines.** Identification of the excreted purine products was accomplished in the following manner. Twenty ml of exponentially growing cells at $6 \times 10^5$ cells per ml were incubated 18 hr in the absence or presence of 10 μM *erythro*-9-[3-(2-hydroxynonyl)]adenine (EHNA). After the 18-hr preincubation, cells were centrifuged and resuspended in 2.5 ml of Eagle's minimal essential medium with 10% dialyzed fetal calf serum, placed in 12-ml Falcon tubes, and incubated with 2 μCi of [U-$^{14}$C]glycine in the absence or presence of 10 μM EHNA. After a 60-min incubation at 37°C with the radioactive glycine, the cells were pelleted by centrifugation. To the supernatant medium was added 1.0 ml of 10% (wt/vol) activated charcoal, and the purines were allowed to adsorb for 10 min at 0°C. The activated charcoal was washed free of most of the contaminating glycine with three 10-ml washes with $H_2O$. The purines were eluted with 5 ml of ethanol/$H_2O$/$NH_4OH$, 5:4:1 (vol/vol), by frequent mixing for 3 hr at 37°C. The charcoal was removed by centrifugation, and the supernatant was blown dry. The purines were dissolved in 40 μl of the ethanol/$H_2O$/$NH_4OH$ mixture, and 5 μl of each sample was spotted onto PEI-cellulose sheets (Macherey–Nagel & Co.). Nucleotides were separated from nucleosides and bases by partition salt chromatography with 0.5 M LiCl for 5 min, 1 M LiCl for 10 min, and 1.5 M LiCl to the top. The hypoxanthine and IMP were resolved on PEI-cellulose with 0.7 M ammonium formate as the developing solvent. Purine bases and nucleosides were resolved by chromatography on PEI-cellulose in *n*-butanol/$CH_3OH$/$H_2O$/$NH_4OH$, 60:20:20:1 (vol/vol). The thin-layer chromatograms were exposed to Kodak XR-5 x-ray film for 3 days and developed.

**Measurement of Intracellular Nucleotide Concentrations and Specific Radioactivities.** Six hundred milliliters of wild-type, AU-100, MPR3-3, and AU-TG50B cells at $\approx 10^6$ cells per ml were incubated with 37.5 μCi of [$^{14}$C]formate (52 mCi/mmol). Cells (200 ml) were harvested at 45 min, 90 min, and 180 min by centrifugation, and the nucleotides were extracted by the method of Khym (30). ATP and GTP were separated by high-performance liquid chromatography with a Partisil-SAX column and a mobile phase of 0.4 M ammonium phosphate, pH 3.4/1.5% acetonitrile at 2.5 ml/min (28). IMP was measured as the ribonucleoside inosine by reverse-phase high-performance liquid chromatography. The acid-soluble extract was injected onto an Altex RP-18 analytical column and eluted with 3% acetonitrile in $H_2O$ at a flow rate of 2.0 ml/min. The effluent was collected between 1.3 and 2.2 min after injection. This fraction contained polar compounds including IMP and excluded nucleosides and other nonpolar compounds. The collected fraction was concentrated to 200 μl under a stream of nitrogen at 60°C. Two units of *Escherichia coli* alkaline phosphatase type III (Sigma) and 25 μl of 0.5 M Tris buffer (pH 9.0) were added, and the sample was incubated at 37°C for 30 min to convert nucleotides to nucleosides. After reacidification with 25 μl of 0.5 M acetic acid, the sample was reinjected onto the Altex RP-18 column, and the nucleosides were eluted with 1.5% acetonitrile/10 mM sodium acetate, pH 4.7. Inosine had a retention time of 10 min and was clearly separated from all other autologous nucleosides. Polar compounds other than nucleotides were not affected by alkaline phosphatase and remained at the solvent front.

Quantities were determined by absorbency at 254 nm and comparisons with standards; radioactivities were determined by liquid scintillation assay of the appropriate eluted fractions.

## RESULTS

The capacities of wild-type and AU-100 cells and their HGPRTase-deficient derivatives to incorporate [$^{14}$C]formate or [$^{14}$C]glycine into intracellular purines are shown in Fig. 1. In nine separate experiments, the adenylosuccinate synthetase-deficient (AU-100) cells incorporated radioactive formate or glycine into intracellular soluble purines at a rate 1/2 to 1/3 that found for wild-type cells (Fig. 1 *A* and *B*) (19). However, examination of the rates of excretion of radiolabeled purine nucleosides into the growth medium indicated that the adenylosuccinate synthetase-deficient cells excreted a large proportion of their newly synthesized purine products (Fig. 1). Conversely, the wild-type cells excreted only small amounts of radioactively labeled purine products into the medium. The total (intracellular plus excreted) rate of incorporation of radioactivity into purines is 3- to 4-fold greater in the AU-100 cell line than in wild type (Fig. 1). Although the specific radioactivities of the intracellular folate precursors of purines were not determined in these experiments, intracellular glycine pools were and did not differ in wild-type and AU-100 cells. Thus, the rate of *de novo* synthesis of IMP, the first purine nucleotide formed and precursor of all other purine metabolites, is increased 3- to 4-fold in the adenylosuccinate synthetase-deficient mutants.

Because the AU-100 cells only slowly incorporate radioactive hypoxanthine from the medium into intracellular nucleotides (19), we thought that the excessive purine nucleoside excretion in the AU-100 cell line might be due to a physiological inhibition of HGPRTase, mediated perhaps by intracellular GMP (19). Therefore, as controls, the rates of incorporation of [$^{14}$C]formate and [$^{14}$C]glycine into purine metabolites were measured for two HGPRTase-deficient clones—one (MPR3-3) isolated from wild-type cells and the other (AU-TG50B) isolated from AU-100 cells (see Fig. 1). The AU-100 cell line excreted much greater quantities of radiolabeled purine metabolites into the culture medium than even the MPR3-3 line did. Moreover, the AU-TG50B line excreted still greater quantities of radiolabel into the medium than did AU-100 cells, and the incorporation of radiolabeled precursors into intracellular purine nucleotides was about 50% greater than that of wild-type and MPR3-3 cells and 2- to 3-fold greater than that of AU-100 cells. These latter observations imply (*i*) that the increased excretion of radiolabeled purine metabolites in adenylosuccinate synthetase deficiency is not due to secondary HGPRTase deficiency and (*ii*) that, in AU-100 cells, HGPRTase is indeed functional, salvaging unlabeled purine metabolites that had accumulated in the culture medium during the normal growth of the cells—i.e., before the addition of radiolabel.

Therefore, we examined the specific radioactivity of ATP, GTP, and IMP in the four cell lines after 45-min, 90-min, and 180-min incubations with radiolabeled formate. The specific radioactivities of ATP and GTP increased linearly for 3 hr in all four cell lines (data not shown, but see legend to Fig. 2). This linearity of radiolabel incorporation into pools of unchanging sizes reflects a steady-state input of radioactivity from a common precursor—i.e., IMP. However, the specific radioactivities of ATP and GTP are highest in AU-TG50B cells, lowest in AU-100, and approximately equal in the wild-type and MPR3-3 cells (data not shown). IMP specific radioactivities were measured and found to have reached a steady state in less than 45 min (data not shown). The IMP specific radioactivity was highest in AU-TG50B, lowest in AU-100, and approximately equal for

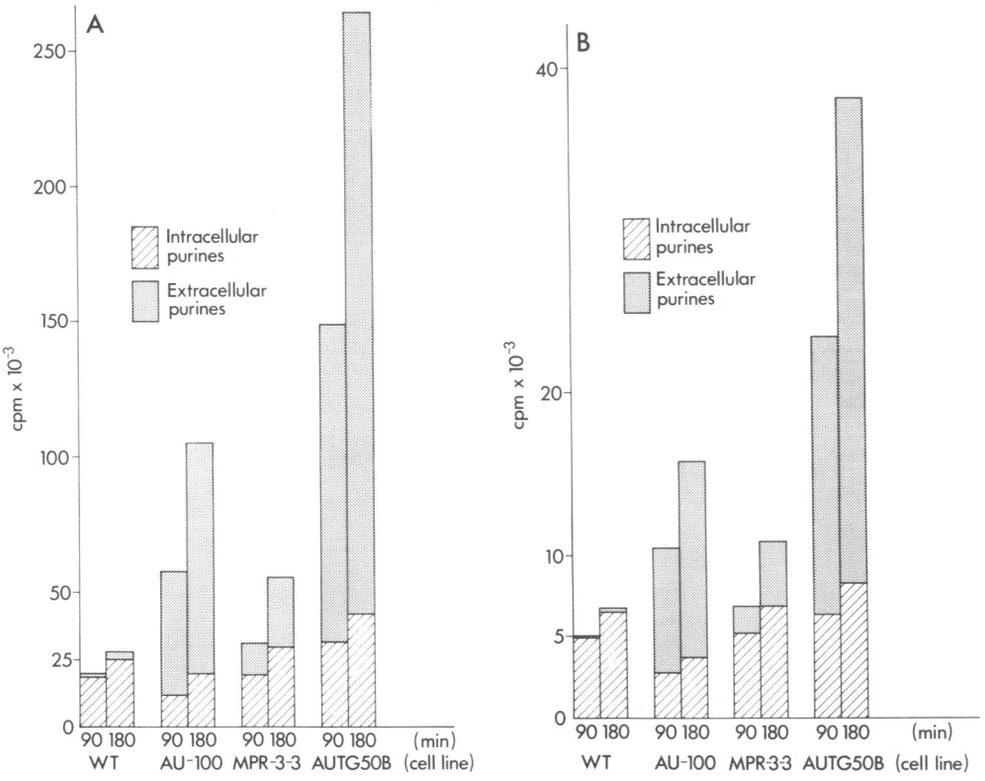


FIG. 1. Rates of radioactive precursor incorporation into intracellular and extracellular purines in wild-type and mutant S49 cells. The rates of [$^{14}$C]formate (52 mCi/mmol) (*A*) or [$^{14}$C]glycine (0.5 mCi/mmol) (*B*) incorporation into intracellular and extracellular purines are indicated for wild-type (WT), adenylosuccinate synthetase-deficient (AU-100), HGPRTase-deficient (MPR3-3), and adenylosuccinate synthetase/HGPRTase-deficient (AU-TG50B) cells. The values reported are those of a typical experiment which has been repeated eight times with either [U-$^{14}$C]glycine or [$^{14}$C]formate precursor with similar results. The incorporation of [$^{14}$C]formate or [U-$^{14}$C]glycine into intracellular and extracellular purines was linear with time to 90 min and with all cell densities used.

both wild-type and MPR3-3 cells (Table 1). Therefore, because IMP-specific radioactivities are known (Table 1) and are at steady state in 45 min, the absolute rates of adenine and guanine nucleotide synthesis (Fig. 2) and inosine excretion (Table 1) can be calculated for all four cell lines. Clearly, the partial deficiency in adenylosuccinate synthetase leads to extraordinary rates of purine nucleoside excretion and to elevated IMP pools, whereas the rates of synthesis of intracellular adenine and guanine nucleotides are slightly reduced and enhanced, respectively, and reflect their relative concentrations in the wild-type and mutant cells (Table 2).

The major purine excretion product of AU-100 and AU-TG50B cells was inosine as shown by the chromatogram in Fig. 3 and by two other chromatographic systems described in *Methods*. Incubation of cells with an adenosine deaminase inhibitor, EHNA, did not alter the profile of excreted purine metabolites in any of the cell types (Fig. 3).

Table 1. Intracellular IMP levels and specific radioactivities and rates of purine metabolite excretion

| Cell line | IMP | | Purine metabolite excretion, |
|---|---|---|---|
| | nmol/$10^9$ cells | cpm/nmol | $\mu$mol/hr·$10^9$ cells |
| Wild type | 4.0 ± 0.9 | 246 ± 143 | 0.05 |
| AU-100 | 18.4 ± 3.7 | 159 ± 33 | 1.49 |
| MPR3-3 | 2.4 ± 0.6 | 305 ± 125 | 0.36 |
| AU-TG50B | 18.1 ± 0.5 | 508 ± 40 | 1.65 |

The average values and standard deviations are those from six determinations for wild-type and AU-100 cells and three determinations for MPR3-3 and AU-TG50B cells. The rates of purine metabolite excretion for each cell line were obtained by dividing the total [$^{14}$C]formate incorporated into extracellular inosine by the specific radioactivity of the IMP pool.

## DISCUSSION

In spite of being deficient in adenylosuccinate synthetase, AU-100 cells maintain near normal intracellular levels of adenine nucleotides. The ability to do so almost certainly results from feedback regulation of *de novo* purine synthesis by adenine nucleotides (10–14). Thus, the levels of intracellular 5-phospho-$\alpha$-D-ribose 1-diphosphate (19) and IMP become secondarily elevated in an attempt to compensate for the partial deficiency of adenylosuccinate synthetase and thereby result in nearly normal ATP levels. As further consequences of the increased IMP pools, guanine nucleotide levels are expanded through an uncompromised [but GMP feedback sensitive (17)] pathway from IMP, and, most strikingly, the excretion of inosine is greatly increased. The massive purine nucleoside excretion into the medium by the AU-100 cell lines seems to be due to the adenylosuccinate synthetase deficiency and not to a secondary deficiency in the purine salvage enzyme, HGPRTase. The relative contributions of the apparent "impaired" hypoxanthine salvage (19) and the adenylosuccinate synthetase deficiency to the excessive purine excretion in the AU-100 cell line have been evaluated by the introduction of a genetic deficiency of HGPRTase into AU-100 cells. The HGPRTase-deficient, adenylosuccinate synthetase-deficient (AU-TG50B) cells overproduce and overexcrete purine metabolites at rates at least as great as do the parental (AU-100) cells and greater than do the cells with the HGPRTase deficiency alone (MPR3-3).

The higher specific radioactivity of the IMP pool in AU-TG50B cells versus AU-100 cells clearly demonstrates that the

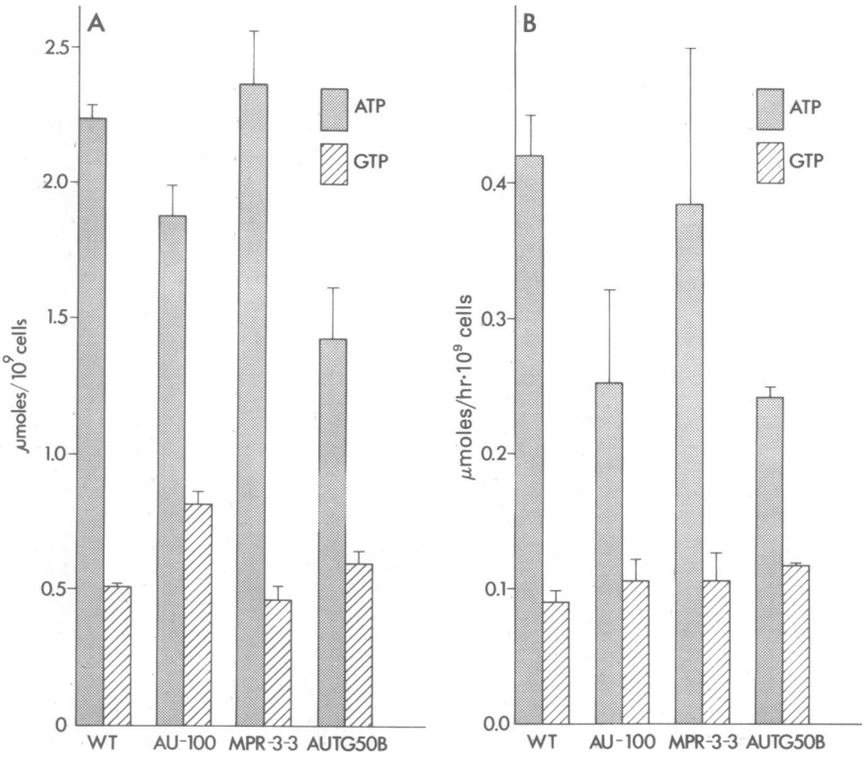


FIG. 2. Measurement of intracellular ATP and GTP concentrations (*A*) and their rates of synthesis (*B*). Cells were incubated with 0.625 μCi/ml of [$^{14}$C]formate (52 mCi/mmol) for 45, 90, and 180 min. ATP and GTP in neutralized $HClO_4$ extracts were separated from other autologous nucleotides by high-performance liquid chromatography, and the radioactivity in each was assayed by liquid scintillation. (*A*) Concentrations of ATP and GTP and their standard deviations ($n = 3$). (*B*) Rates of synthesis of ATP and GTP and their standard deviations ($n = 3$), utilizing the values in Table 1 for the specific radioactivity of IMP, the precursor of both ATP and GTP. The nucleoside triphosphates account for at least 90% of all adenine and guanine nucleotides in S49 cells.

salvage of nonradioactive extracellular inosine excreted by AU-100 cells during the 18-hr preincubation dilutes the IMP radioactivity during the labeling procedures. The specific radioactivities of the ATP and GTP pools in the various cell lines reflect the activity of the salvage process (responsible for isotope dilution) in those particular cells. These observations, plus the facts that the IMP pool of the HGPRTase-deficient (MPR3-3) cells is not elevated as it is in AU-100 cells (ref. 19; Table 1) and that an adenosine deaminase inhibitor does not alter the nature of the excreted purines, implicate the elevated IMP pool in the massive purine excretion by the adenylosuccinate synthetase-deficient cells. The salvage of inosine (through hypoxanthine) from the medium by AU-100 cells can account for the (apparent) impaired salvage of radioactive hypoxanthine.

It is interesting to note that a 4-fold elevation in intracellular IMP in AU-100 cells causes a 30- to 50-fold increase in *de novo* purine excretion. Presumably, elevated IMP activates or induces IMP dephosphorylation, which leads to the excessive loss of inosine to the medium.

Table 2. Relative concentrations and rates of synthesis of ATP and GTP

| Cell line | GTP/ATP ratio | |
|---|---|---|
| | Intracellular concentrations | Rates of synthesis |
| Wild type | 0.23 | 0.21 |
| AU-100 | 0.43 | 0.42 |
| MPR3-3 | 0.20 | 0.28 |
| AU-TG50B | 0.42 | 0.48 |

The ratios of intracellular nucleotide concentrations and of their rates of synthesis are derived directly from the data in Figs. 1 and 2.

Previous studies of the AU-100 cells in this laboratory (19) failed to detect their excretion of large quantities of inosine. This failure was due to the fact that the deproteinized culture medium was not subject to acid hydrolysis to remove the ribosyl moiety from purine metabolites before precipitation of the purine base by silver; any substituent on the N-9 of a purine prevents its precipitation by this metal ion (unpublished observations). Also in previous studies conducted 3 years ago (19), we observed absolute concentrations of GTP lower than those re-

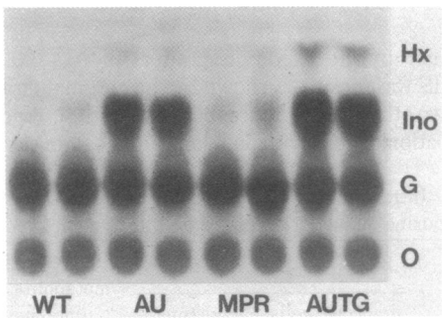


FIG. 3. Identification of purines excreted by S49 cells. The radioactive purines excreted into the culture medium were isolated by adsorption to activated charcoal and chromatographed on PEI-cellulose thin layers. The positions of hypoxanthine (Hx), inosine (Ino), and [U-$^{14}$C]glycine (G) after chromatography in *n*-butanol/$CH_3OH$/$H_2O$/$NH_4OH$, 60:20:20:1 (vol/vol) are indicated, as is the origin (O). A track for wild-type (WT), adenylosuccinate synthetase-deficient (AU), HGPRTase-deficient (MPR), and adenylosuccinate synthetase/HGPRTase-deficient (AUTG) cells incubated in the absence (left member of each pair of tracks) or presence (right member of each pair of tracks) of 10 μM EHNA is shown.

ported in the current studies, even though the cells used for all experiments reported herein were freshly thawed from an original frozen stock. These differences in absolute concentrations are of unknown cause but perhaps related to differences in horse sera.

Pharmacologic simulation of adenylosuccinate synthetase deficiency in a human lymphoblastoid cell line in culture is reported to enhance excretion of purines by these cells (31). This corroborates the causal role of adenylosuccinate synthetase deficiency in the overexcretion of purine nucleosides by AU-100 cells. Green and Ishii (32) have proposed that an imbalance in the AMP and GMP portions of the purine synthetic pathway would cause purine overproduction because the conversion of GMP to AMP is ineffectual in mammalian cells. Their prediction is borne out.

In a human with a partial deficiency in adenylosuccinate synthetase one would expect peripheral tissues to excrete excessive amounts of newly synthesized purines as inosine, which would be rapidly metabolized to uric acid. Therefore, it is possible that among the population of hyperuricemic patients are individuals with genetically reduced activity of adenylosuccinate synthetase; a complete deficiency would almost certainly be lethal. Thus, humans heterozygous for adenylosuccinate synthetase deficiency might inherit hyperuricemia in a dominant fashion.

**Note Added in Proof.** Repeat assays of adenylosuccinate synthetase catalytic activity with radioactively labeled IMP, rather than aspartate (19), confirm an 80–90% deficiency in the AU-100 cells and demonstrate increased lability of their residual activity. Experiments in which extracts of AU-100 and wild-type cells were mixed in different proportions generate the predicted additive levels of activity, thus eliminating the likelihood of a diffusible enzyme inhibitor in AU-100 cells.

This work was supported by a grant from the National Institutes of Arthritis, Diabetes, and Digestive and Kidney Diseases (AM-20428). D.W.M. is an Investigator of the Howard Hughes Medical Institute.

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