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Biochemistry

Purine metabolism in cultured human fibroblasts derived from patients deficient in hypoxanthine phosphoribosyltransferase, purine nucleoside phosphorylase, or adenosine deaminase

(Lesch-Nyhan syndrome/immunodeficiency)

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ABSTRACT **Rates of purine synthesis *de novo*, as measured by the incorporation of [^{14}C]formate into newly synthesized purines, have been determined in cultured human fibroblasts derived from normal individuals and from patients deficient in adenosine deaminase, purine nucleoside phosphorylase, or hypoxanthine phosphoribosyltransferase, three consecutive enzymes of the purine salvage pathway. All four types of cell lines are capable of incorporating [^{14}C]formate into purines at approximately the same rate when the assays are conducted in purine-free medium. The purine overproduction that is characteristic of a deficiency in either the transferase or the phosphorylase and that results from a block in purine reutilization can be demonstrated by the resistance of [^{14}C]formate incorporation into purines to inhibition by hypoxanthine in the case of hypoxanthine phosphoribosyltransferase-deficient fibroblasts and by resistance to inhibition by inosine in the case of purine nucleoside phosphorylase-deficient fibroblasts.**

Human cells in culture utilize purines derived from two metabolic routes—either by synthesis *de novo* beginning with 5-phosphoribosyl-1-pyrophosphate (PP-ribose-P) and glutamine or by "salvage" from PP-ribose-P and purines present in the medium or arising from the intracellular degradation of nucleic acids. Individuals who lack purine nucleoside phosphorylase (PNP) or hypoxanthine phosphoribosyltransferase (HPRT), two consecutive enzymes of the purine salvage pathway, synthesize purines *de novo* at an accelerated rate (1–3). Although several cell culture systems have been devised to demonstrate the purine overproduction characteristic of patients with a deficiency of HPRT (4–8), none has been devised to show the comparable overproduction in PNP deficiency. The mechanism by which the rate of purine synthesis *de novo* is controlled has been the subject of intensive study by numerous investigators. It is believed to involve the regulation of PP-ribose-P synthetase or PP-ribose-P amidotransferase, the enzymes which catalyze the first two reactions of this pathway. In Ehrlich ascites tumor cells, the rate of synthesis of PP-ribose-P has been shown to be inversely related to the concentration of adenine and guanine nucleotides (9). Partially purified human PP-ribose-P synthetase has been shown to be inhibited by ADP and AMP (10). The activity of PP-ribose-P amidotransferase and of the *de novo* pathway as a whole may be controlled by the intracellular levels of PP-ribose-P and of adenine and guanine nucleotides (4, 11–16). PP-ribose-P is normally present at concentrations well below the K_m for the enzyme. It also converts the human enzyme to its more active, low molecular weight form. Adenine and guanine nucleotides act as inhibitors of the partially purified enzyme and also convert the human enzyme to its less active, high molecular weight form. Thus, either an increase in the concentration of PP-ribose-P or a decrease in the levels of inhibitory nucleotides could potentially accelerate the rate of purine synthesis *de novo* and result in purine "overproduction." This communication concerns the study of purine metabolism in cultured human fibroblasts deficient in each of three enzymes of the purine salvage pathway and attempts to define better a "normal" rate of purine synthesis *de novo* as compared to purine "overproduction." In particular, we have attempted to rectify the previously reported discrepancy in rates of purine synthesis *de novo* in patients deficient in PNP and in their cultured cells (1).

The three enzyme deficiencies studied are: HPRT, PNP, and adenosine deaminase (ADA). A lack of the enzyme HPRT (17) leads to the Lesch-Nyhan syndrome (2) with its characteristic neurological dysfunction and purine overproduction *de novo* resulting in pronounced hyperuricemia. A lack of PNP (18) results in an immunodeficiency disease with a specific defect in T-cell function as well as purine overproduction *de novo*; however, hypouricemia rather than hyperuricemia is seen because purine catabolism stops with the formation of purine nucleosides. Large quantities of inosine and guanosine and smaller amounts of the corresponding deoxynucleosides are excreted in the urine (1, 3). ADA deficiency leads to a severe combined immunodeficiency with T-cell and usually B-cell dysfunction (19). No purine overproduction has been found in patients lacking ADA (20).

MATERIALS AND METHODS

Source and Growth of Cells. Fibroblasts were grown from foreskins obtained from apparently normal male infants or from skin biopsies obtained from normal volunteers or from patients deficient in HPRT, ADA (20), or PNP (3). Fibroblast cultures were routinely maintained in Eagle's minimal essential medium supplemented with 10% fetal calf serum, 2 mM glutamine, penicillin (100 units/ml), and streptomycin (100 μg/ml). In some experiments, the medium was supplemented with fetal calf serum that had been extensively dialyzed against 0.9% NaCl. Low serum medium contained AutoPow minimal essential medium (Flow Laboratories), supplemented with 0.1% fetal calf serum, 2 mM glutamine, penicillin (100 units/ml), streptomycin (100 μg/ml), 4% bovine serum albumin, transferrin (5 μg/ml), and 4% (vol/vol) Coon's F-12 medium (minus hypoxanthine, thymidine, and folic acid) (21).

Rate of Purine Synthesis *De Novo*. Confluent fibroblasts

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Abbreviations: PP-ribose-P, 5-phosphoribosyl 1-pyrophosphate; HPRT, hypoxanthine phosphoribosyltransferase; PNP, purine nucleoside phosphorylase; ADA, adenosine deaminase; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

were trypsinized, plated at a density of approximately 3×10^5 cells per 35-mm dish in minimal essential medium containing 10% dialyzed fetal calf serum, and allowed to grow in a 37° incubator maintained at 5% CO_2 and 95% air for 15–20 hr. Two or 3 hr prior to the initiation of the experiment, the cells were washed three times with medium supplemented with 40 mM sodium 4-(2-hydroxyethyl)-1-piperazineethanesulfonate (Hepes), pH 7.4, and any appropriate additions, overlaid with 0.6 ml of the same medium, and allowed to equilibrate in 5% CO_2 and 95% air. The assay was begun by the addition of 5 μCi of [^{14}C]formate (60 μCi/μmol) per dish and terminated by aspiration of the medium and subsequent addition of 1 ml of 0.4 M $HClO_4$ to extract the intracellular purines. In some experiments the newly synthesized purines excreted into the medium were also quantitated after precipitation of protein with 0.4 M $HClO_4$. In either case, the deproteinized solution was heated to 100° for 1 hr, and the free purine bases were separated by chromatography on Dowex-50 and quantitated by liquid scintillation counting as described by Hershfield and Seegmiller (22). Each time point required three dishes: two for duplicate determination of the amount of [^{14}C]formate incorporated into total acid-soluble intracellular purines and one for the determination of cell numbers.

Cell Extracts and Enzyme Assays. ADA was assayed by a modification of the procedure of Snyder *et al.* (23) but with 100 mM Tris·HCl (pH 7.0) as buffer, which eliminated the further conversion of the product inosine to hypoxanthine. PNP activity was measured by the same procedure, but with 100 mM sodium phosphate (pH 7.4) as buffer and 300 μM [8-^{14}C]inosine as substrate. Protein concentrations were determined by the method of Lowry *et al.* (24).

Cell extracts were prepared from one 75-cm^2 flask of confluent fibroblasts. The cells were washed three times with a buffer containing 0.04 M sodium Hepes (pH 7.4), 0.13 M NaCl, 0.4% bovine serum albumin, 100 μM $MgCl_2$, and 10 μM $CaCl_2$, and were lysed by freezing and thawing three times in liquid nitrogen. The flask was centrifuged for 3 min at 1500 rpm and the supernatant was removed and centrifuged for 1 hr at 20,000 rpm.

Chemicals. Radiochemicals were purchased from Amersham/Searle Corp.: sodium [^{14}C]formate (60 μCi/μmol), [2-^{3}H]adenosine (20 Ci/mmol), and [8-^{14}C]inosine (60 mCi/mmol). All other chemicals were of the highest quality available from commercial sources.

RESULTS

The incorporation of [^{14}C]formate into total intracellular acid-soluble purines by subconfluent fibroblasts was linear with respect to time for at least 90 min; duplicate determinations agreed within 10% (data not shown). Less than 10% of the total counts incorporated were found in acid-insoluble material (data not shown). The rates of incorporation of [^{14}C]formate into intracellular purines by normal, HPRT$^-$, PNP$^-$, and ADA$^-$ fibroblasts in medium supplemented with 10% dialyzed fetal calf serum, and for HRPT$^+$ fibroblasts supplemented with 10% undialyzed fetal calf serum are shown in Fig. 1. Although the average rate of incorporation is 68% higher in HPRT$^-$ fibroblasts than in normal fibroblasts, the ranges in all four cell types overlap to such a degree (when the assays are conducted in medium supplemented with dialyzed fetal calf serum), that it is not possible to identify the presence of an enzyme defect in a cell line by the cells' rate of [^{14}C]formate incorporation into purines alone. This is in contrast to the rates obtained for HPRT$^+$ fibroblasts when the experiments are carried out in medium containing undialyzed fetal calf serum (Fig. 1); in this

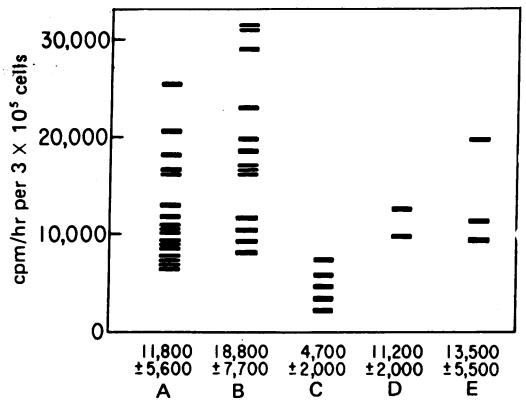

FIG. 1. Rates of incorporation of [^{14}C]formate into acid-soluble intracellular purines by normal, HPRT$^-$, PNP$^-$, and ADA$^-$ fibroblasts. The method is the standard assay as described in *Materials and Methods* with medium supplemented with either 10% dialyzed (columns A, B, D, and E) or undialyzed fetal calf serum (column C). The results are a compilation of data obtained from multiple assays using three normal fibroblast lines, four HPRT-deficient lines, one PNP-deficient line, and two ADA-deficient lines. The mean ±SD for each group of assays is indicated at the bottom of the appropriate column. Columns: A, normal; B, HPRT$^-$; C, HPRT$^+$; D, ADA$^-$; E, PNP$^-$.

case, the rate of incorporation is 4–5 times greater for HPRT$^-$ cells. PNP$^-$ and ADA$^-$ fibroblasts showed rates of [^{14}C]formate incorporation that were diminished to the same extent as in control cells when the assays were conducted in medium supplemented with 10% undialyzed fetal calf serum (data not shown). In all four types of cell lines, the rate of incorporation of [^{14}C]formate was increased by an additional 25–60% when the amount of newly synthesized purines excreted into the medium was also examined. The proportion of newly synthesized purines excreted in the medium was approximately the same in all four types of cell lines, but varied from experiment to experiment. Data from a representative experiment involving normal and HPRT$^-$ fibroblasts are shown in Fig. 2.

The effect of hypoxanthine on the rate of incorporation of [^{14}C]formate into purines in normal and HPRT-deficient fibroblasts is shown in Fig. 3. Significant inhibition can be seen in the control fibroblasts with concentrations as low as 1 μM, whereas HPRT$^-$ fibroblasts are not inhibited by even 50 μM hypoxanthine.

In an attempt to increase the rate of [^{14}C]formate incorpo-

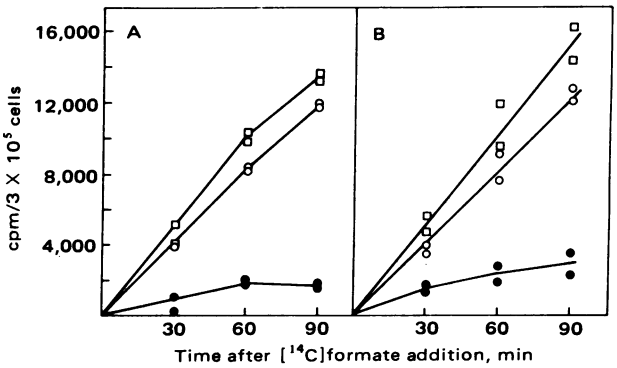

FIG. 2. Incorporation of [^{14}C]formate into acid-soluble purines by normal and HPRT$^-$ fibroblasts in medium supplemented with 10% dialyzed fetal calf serum. O, Intracellular purines; ●, purines excreted into the medium; □, total purines. (*A*) Normal (HPRT$^+$) fibroblasts. (*B*) HPRT$^-$ fibroblasts.

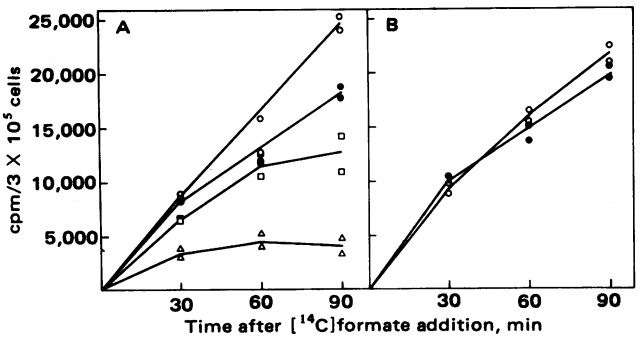

FIG. 3. Effect of hypoxanthine on the incorporation of [^{14}C]formate into acid-soluble intracellular purines by normal and HPRT$^-$ fibroblasts. Medium supplemented with 10% dialyzed fetal calf serum was used in the standard assay. Hypoxanthine was added at the beginning of the equilibration period, immediately after the cells were washed. (*A*) Normal fibroblasts. ○, No hypoxanthine; ●, 1 μM hypoxanthine; □, 3 μM hypoxanthine; △, 10 μM hypoxanthine. (*B*) Lesch–Nyhan fibroblasts. ○, No hypoxanthine; ● 50 μM hypoxanthine.

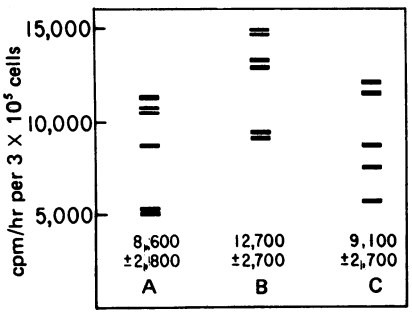

FIG. 4. Rates of incorporation of [^{14}C]formate into acid-soluble intracellular purines by normal, PNP$^-$, and ADA$^-$ fibroblasts. The method is the standard assay as described in *Materials and Methods* for medium supplemented with 0.1% fetal calf serum. The results are a compilation of data obtained from multiple assays using three normal fibroblast lines (A), one PNP-deficient line (B), and two ADA-deficient lines (C). The mean ±SD for each group of assays is indicated at the bottom of the appropriate column.

ration into purines in Lesch–Nyhan fibroblasts, the assays were conducted in the presence of 16 mM phosphate and either 50 μM inosine or 50 μM methylene blue, conditions which have been shown by others to increase intracellular levels of PP-ribose-P in human erythrocytes (25), fibroblasts (26, 27), or Ehrlich ascites cells (28). However, none of the treatments increased the rate of [^{14}C]formate incorporation (data not shown).

In order to study purine synthesis *de novo* in PNP$^-$ and ADA$^-$ cells under conditions where the enzyme defects exhibited by these cells are not corrected by the substantial quantities of PNP and ADA found in fetal calf serum, we developed a growth medium that contained only 0.1% fetal calf serum (see *Materials and Methods*). The quantities of PNP and ADA present in medium supplemented with 10% fetal calf serum are comparable to the levels found in normal fibroblasts, and they are at least 100 times higher than the activities found in fibroblasts from patients deficient in PNP and ADA (Table 1). Use of a medium that contains only 0.1% fetal calf serum substantially reduces the extracellular concentrations of ADA and PNP. Fibroblasts were grown in the low serum medium for at least 5 days prior to the determination of their rate of [^{14}C]formate incorporation into purines. The results for normal, ADA$^-$, and PNP$^-$ fibroblasts are shown in Fig. 4. By using this medium, PNP$^-$ and normal fibroblasts could be distinguished based upon the degree of inhibition of [^{14}C]formate incorporation into purines caused by 50 μM inosine; the PNP$^-$ fibroblasts were highly resistant (Fig. 5). Adenosine (50 μM) caused virtually total inhibition of [^{14}C]formate incorporation into purines in both control and PNP$^-$ fibroblasts (data not shown). Adenosine, inosine, or hypoxanthine (each at 50 μM) also caused complete inhibition of [^{14}C]formate incorporation in ADA$^-$ fibroblasts (data not shown).

Table 1. Contributions of medium and cells towards total ADA and PNP activity under conditions of the *de novo* purine synthesis assay

Enzyme source	ADA, units*/assay	PNP, units*/assay
Medium with 10% fetal calf serum†	26	31
Medium with 0.1% fetal calf serum†	0.26	0.31
Normal fibroblasts‡	16	59
PNP$^-$ fibroblasts‡	—	0.15
ADA$^-$ fibroblasts‡	<0.08	—

* One unit = 1 nmol/hr.
† Enzyme activity in 0.6 ml of medium.
‡ Enzyme activity in 3 × 10^5 cells.

DISCUSSION

Determination of the rates of incorporation of [^{14}C]formate into total acid-soluble intracellular purines by normal, HPRT$^-$, PNP$^-$, and ADA$^-$ fibroblasts shows that these cells are all capable of similar rates of incorporation when the assays are carried out in purine-free medium—i.e., in medium containing 10% dialyzed fetal calf serum. These results confirm in fibroblasts the findings of Hershfield and Seegmiller (29) for normal and HPRT-deficient lymphoblasts. However, normal, PNP$^-$, and ADA$^-$ fibroblasts, but not HPRT$^-$ fibroblasts, show substantially lower rates of [^{14}C]formate incorporation when the assays are performed in medium supplemented with 10% undialyzed fetal calf serum, and thus appear to be inhibited by some component of undialyzed fetal calf serum. This inhibitory substance in fetal calf serum is probably hypoxanthine, because only in HPRT$^-$ fibroblasts is the incorporation of [^{14}C]formate into purines resistant to inhibition by hypoxanthine. Therefore, in the case of Lesch–Nyhan fibroblasts, purine "overproduction" can be better defined as resistance to the inhibition normally produced by hypoxanthine.

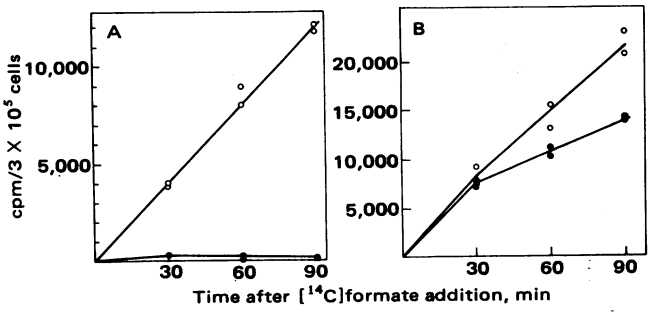

FIG. 5. Effect of inosine on the incorporation of [^{14}C]formate into acid-soluble intracellular purines by normal and PNP$^-$ fibroblasts. The medium was supplemented with 0.1% fetal calf serum and inosine was added at the beginning of the equilibration period, immediately after the cells were washed. ○, No inosine; ●, 50 μM inosine. (*A*) Normal fibroblasts. (*B*) PNP$^-$ fibroblasts.

The inhibition of [^{14}C]formate incorporation into purines in HPRT$^+$ cells by hypoxanthine probably accounts for the discrepancy in our results and those of other workers (4–8) who have reported a 2- to 10-fold increase in the relative rates of purine synthesis *de novo* in HPRT-deficient as compared to normal fibroblasts or lymphoblasts. Their experiments were done in medium containing either undialyzed fetal calf serum or no serum at all. Hershfield and Seegmiller (29) have shown that serum-free medium causes lysis of lymphoblasts which could release purines into the medium in concentrations sufficient to inhibit purine synthesis *de novo* in normal cells, particularly if the cells have been resuspended at a high density. We have made similar observations of leakage of substantial quantities of hypoxanthine and other purines (data not shown) into serum-free medium by fibroblasts resuspended at a density of $1–2 \times 10^7$ cells per ml. A much smaller, but still significant amount of leakage even occurred when the cells were resuspended at high density in our 0.1% fetal calf serum medium. This leakage may be responsible for the higher average rate of [^{14}C]formate incorporation into newly synthesized purines observed in Lesch–Nyhan fibroblasts, because the presence of only 1 μM hypoxanthine will partially inhibit *de novo* purine synthesis in HPRT$^+$ cells.

The resistance of [^{14}C]formate incorporation into purines to inhibition by hypoxanthine in Lesch–Nyhan cells can be explained in at least two ways: first, PP-ribose-P concentrations will not be lowered by reaction with hypoxanthine to form IMP and, second, there may also be lowered concentrations of nucleotides inhibitory to the purified amidotransferase (15) because the salvage pathway is not operational. The physiological significance of this latter mechanism is not known. Henderson and colleagues (9, 30) have found no alterations in amidotransferase activity in intact Ehrlich ascites cells under conditions in which the levels of the adenine and guanine nucleotides have been elevated or decreased. Brenton *et al.* (31) and others (4, 32) have measured the intracellular concentrations of purine nucleotides in normal and HPRT-deficient fibroblasts and lymphoblasts and have found no evidence for decreased concentrations of adenine and guanine nucleotides in cells that lack HPRT. However, the concentrations of AMP and GMP, the most potent inhibitors of the amidotransferase, have not been precisely determined because they are very close to the detection limits of the methods available. Furthermore, the existence of separate compartmentalized pools of adenine and guanine nucleotides that may be inhibitory to the amidotransferase must be considered. Additional experiments need to be designed which will allow alterations in the intracellular concentrations of potentially inhibitory nucleotides independently from changes in the concentration of PP-ribose-P, so that the relative importance of these two factors in controlling the rate of purine synthesis *de novo* may be evaluated. Our finding that neither methylene blue nor inosine increased the rate of [^{14}C]formate incorporation into purines in Lesch–Nyhan cells is consistent with Hershfield's (29) findings that indicate that there may be conditions where the concentration of PP-ribose-P is not rate-limiting for purine synthesis *de novo* (12, 27).

At the present time we are unable to explain the wide range of values obtained for the rates of [^{14}C]formate incorporation into purines by normal or Lesch–Nyhan fibroblasts, even when the same cell line is used in several experiments. However, the variation does not appear to be due to passage number, cell density, length of the equilibration period, or the length of time between plating the cells and the initiation of the experiments, because alterations in these parameters were carefully recorded and controlled. Furthermore, there was no loss in cell viability during the course of the experiment. The large scatter in the data demonstrates the need for several experiments before any conclusions can be drawn regarding differences in the rates of [^{14}C]formate incorporation into purines among various cell lines. It is possible that fluctuations in intracellular concentrations of the folate coenzymes could account for some of the variation seen.

Our studies of [^{14}C]formate incorporation into purines in PNP-deficient fibroblasts may explain the previous report of Martin *et al.* (1) that PNP$^-$ cells synthesize purines *de novo* at a low rate compared to Lesch–Nyhan fibroblasts, and thus do not constitute an appropriate model for the purine overproduction seen in patients who are deficient in PNP. Martin's experiments were conducted in medium supplemented with 10% undialyzed fetal calf serum; it is to be expected that purine synthesis *de novo* should be inhibited under these conditions, because PNP$^-$ cells still have functional HPRT and are therefore sensitive to inhibition by the hypoxanthine in the fetal calf serum. In PNP-deficient fibroblasts, purine overproduction can be demonstrated by their resistance to inhibition by exogenous inosine.

Our finding that adenosine, inosine, and hypoxanthine inhibit the incorporation of [^{14}C]formate into purines by ADA$^-$ fibroblasts is consistent with the observation that patients who lack ADA do not overproduce purines *de novo* (20) and suggests that adenosine can be effectively metabolized via adenosine kinase. In conclusion, we feel that purine overproduction in the above mutations can best be demonstrated by the resistance of purine synthesis *de novo* to potentially inhibitory purine nucleosides and bases, rather than by the absolute rate of incorporation of [^{14}C]formate into purines.

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