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Phosphate-Induced Phosphoribosylpyrophosphate Elevations to Assess Deranged Folate and Purine Nucleotide Metabolism[1] (42590)

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Abstract. Phosphoribosylpyrophosphate (PRPP) levels increase several-fold in HL-60 cells adapted to folate deficiency either by continuous passage in folate-deficient medium or by short-term incubation with 10^{-8} *M* methotrexate (MTX). The addition of folic acid (PteGlu) or 5-formyltetrahydrofolic acid (5-CHO-H_4PteGlu) in the form of Leucovorin normalizes this effect. The reactions for measuring PRPP levels are time and temperature dependent and are influenced by PRPP-reacting substances in undialyzed serum. Inorganic phosphate (PO_4), when added to the assay, markedly stimulates PRPP levels in HL-60 cells and can be used to stress folate-dependent PRPP utilization for purine synthesis. The integrity of the folate-dependent pathways of purine-synthesizing cells can be sensitively assessed by measurement of PRPP levels during a 2-hr assay in the presence of PO_4 in medium free of folate but containing dialyzed serum. In HL-60 cells that are folate deficient or in the presence of MTX (as low as 2×10^{-9} *M*), PO_4-stimulated PRPP levels remain elevated due to ineffective utilization unless folate is added to the incubation mixture. The sensitivity of this PRPP assay to metabolically assess the integrity of folate-dependent reactions in purine synthesis is comparable to that of the deoxyuridine suppression assay. Inorganic phosphate can also be used to stimulate the incorporation of purine analogs, such as 6-mercaptopurine, into intact red blood cells which may have therapeutic implications for targeting drug delivery.

Phosphoribosylpyrophosphate (PRPP) is a precursor of purine nucleotides, a regulator of the rate of purine nucleotide synthesis, and a participant in the 10-step pathway to inosinate (IMP) (1, 2). Formate is required in two of these steps and is provided by the formyl group of 10-formyltetrahydrofolate; thus, folate may become a limiting metabolite in purine synthesis. Folate-deficient, actively purine-synthesizing cells would be expected to show elevated PRPP concentrations as a result of piling-up and of decreased negative feedback from purine nucleotides. Elevated PRPP has been found in L1210 cells exposed to methotrexate (MTX) (3). PRPP levels increase in several human malignant cell lines cultured in media containing low levels of folic acid (PteGlu, 10–25 n*M*) as compared with replete media (2.3 μM) (4). The elevation of PRPP correlates with a decrease in intracellular folate levels and development of megaloblastosis (manuscript in preparation). High concentrations of inorganic phosphate (PO_4) stimulate PRPP synthetase activity and elevate PRPP in red blood cells (RBC) (5). PRPP is also remarkedly elevated in HL-60 cells assayed following incubation with high PO_4 concentrations (6). We now report that a PO_4-induced PRPP intracellular load can stress the integrity of the folate-dependent pathways for the synthesis of IMP to utilize PRPP and can be used as a sensitive assay of folate-dependent pathways in purine synthesizing cells.

Materials and Methods. HL-60 (ATCC No. CCL 240) is a promyelocytic cell line derived from a patient with acute promyelocytic leukemia (7). The cells were maintained at 37°C in a humidified 7.5% CO_2 environment as a continuous suspension culture using RPMI 1640 (2.3 μM with respect to PteGlu) containing 15% heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were initially suspended at a concentration of 2×10^5 cells/ml and passaged twice weekly. Cells were depleted of folate by substituting RPMI 1640 containing 25 n*M* PteGlu. Folate deficiency was char-

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acterized by megaloblastosis, a decrease in the doubling time, and an accompanying abnormal deoxyuridine suppression test correctable by PteGlu as measured by the method of Metz *et al.* (8). Adaptation was described by a disappearance of megaloblastosis and a normalizing of the growth rate while maintaining the abnormal deoxyuridine suppression. All tissue culture reagents were purchased from GIBCO (Grand Island, NY). When dialyzed FBS was used, the serum was dialyzed against 0.15 *M* NaCl until there was less than 0.005% glucose. Cells were harvested for study during exponential growth. In some experiments, the cells were centrifuged and washed to remove PRPP-reacting substances present in the cell culture medium with 1 ml Tris–EDTA buffer (10 m*M* Tris, 1 m*M* EDTA, pH 7.4). Cells ($5–8 \times 10^6$) were pipetted into disposable borosilicate test tubes. MTX or folates were added followed by 1 *M* potassium PO_4 buffer (pH 7.5) to a final concentration of 20 m*M*, according to the experimental protocol, and incubations were carried out at 37°C in a CO_2 environment. At the end of the incubation, the tubes were centrifuged at 1000 rpm for 5 min at 4°C. The supernatants were discarded and the cell pellets were resuspended in 1 ml Tris–EDTA buffer and stored at −35°C until assayed. MTX (sodium salt), 5-formyltetrahydrofolic acid (5-CHO-H_4PteGlu; Leucovorin), and PteGlu (Folvite) were from Lederle Laboratories, (Pearl River, NY). 6-Mercaptopurine (6-MP) was from Sigma (St. Louis, MO). All other reagents were Fisher certified (Fisher Scientific, Piscataway, NJ). PRPP was assayed by counting the $^{14}CO_2$ liberated when orotidylate decarboxylase (ODC) acts on orotidylate (formed by the reaction between PRPP and [*carboxyl*-^{14}C]orotate) catalyzed by orotate phosphoribosyltransferase (OPRT) by the method of Tax and Veerkamp (9) and modified by us for nonerythroid cells (10). The cell extracts were freeze-thawed three times, immersed in boiling water for 45 sec, and immediately chilled in ice water for not less than 30 sec. The boiled cell extracts were assayed directly or after appropriate dilution. $^{14}CO_2$ was liberated by acidification with H_2SO_4, trapped in NCS reagent (Amersham, Arlington Heights, IL), and counted in toluene/Liquifluor (New England Nuclear, Boston, MA) to a counting error of 0.2%. Values were expressed as picomoles per 10^6 cells as referenced to a standard curve. All experiments were performed in triplicate and variations were less than 10%. PRPP and ODC + OPRT (mixed enzymes) were from Sigma. [*carboxyl*-^{14}C]-Orotate (60 mCi/mmole) was purchased from New England Nuclear and diluted with cold orotate (Sigma).

In studies utilizing RBC for loading with 6-MP, 5 μl of RBC was incubated for 60 min in isotonic Tris–NaCl, pH 8, containing PO_4 (20 m*M*) and 0.5% glucose in order to stimulate erythrocytic PRPP. Thereafter, the RBC were washed with Tris–NaCl containing 0.5% glucose without PO_4 and incubated with increasing amounts of 6-MP for 30 min in a volume of 20 μl. Washing and incubating the RBC in the absence of PO_4 serve to eliminate further stimulation of PRPP synthesis and when 6-MP is added only PRPP utilization is measured. The RBC were obtained from freshly drawn anticoagulated human blood donated to the Mount Sinai Hospital Blood Bank and informed consent was obtained according to the guidelines set by the declaration of Helsinki.

Results. The PRPP levels in HL-60 cells cultured in folate-deficient media (25 n*M* PteGlu) were much higher than those of HL-60 cells cultured in the standard folate-replete media (2.3 μ*M* PteGlu) (Fig. 1). In the presence of increasing levels of PO_4 there was a concentration-dependent elevation of PRPP levels in HL-60 cells whether cultured in folate-replete or -deficient media. At all PO_4 concentrations studied there continued to be a greater

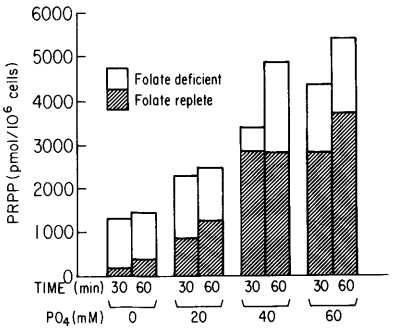

FIG. 1. PRPP levels in HL-60 cells cultured in folate-replete (2.3 μ*M* PteGlu) and folate-deficient (25 n*M* PteGlu) media. Cells were incubated for 30 and 60 min with 0–60 m*M* PO_4.

PRPP level in HL-60 cells cultured in folate-deficient media. However, these differences were less obvious when the cells were incubated in the presence of PO_4 at concentrations greater than 40 m*M*. Following an initial increase in PRPP in the presence of 20 m*M* PO_4 there was a decline toward baseline levels when HL-60 cells were grown in folate-replete media (Fig. 2). In contrast, the PRPP levels in HL-60 cells grown in folate-deficient media remained elevated when continuously incubated with 20 m*M* PO_4 over a 3-hr period. There was a fall to baseline PRPP levels when PteGlu was added to the folate-depleted HL-60 cells.

The PO_4-induced elevated PRPP levels in the HL-60 cells declined when small amounts of FBS (but not dialyzed FBS) were added to the incubation mixture (Fig. 3). FBS (20 μl) contained approximately 2.5 nmole PRPP-reacting substances (presumably hypoxanthine) (10). A parallel 30-min incubation with increasing amounts of a known PRPP-reacting metabolite (i.e., adenine) resulted in a comparable decline of PRPP when cells were incubated with as little as 2 nmole adenine (data not shown). Consequently, the HL-60 cells were washed free of PRPP-reacting substances present in the tissue culture medium as outlined under Materials and Methods. Under these conditions, the PRPP elevations induced by PO_4 (20 m*M*) were again greater in the folate-depleted HL-60 cells than in the folate-replete HL-60 cells and remained elevated unless folate was added to the incubation mixture (Fig. 4). In most experiments, 5-CHO-H_4PteGlu (4 μM) was more effective than PteGlu (100 μM) in utilizing the PRPP and returning the PRPP to baseline levels in folate-depleted cells.

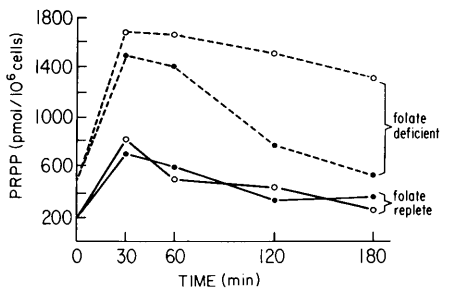

FIG. 2. PRPP levels in HL-60 cells incubated with 20 m*M* PO_4. Cells were cultured in folate-replete (2.3 μM PteGlu) and folate-deficient (25 n*M* PteGlu) media. At 0 time, 20 m*M* PO_4 was added with (●) and without (○) 100 μM PteGlu.

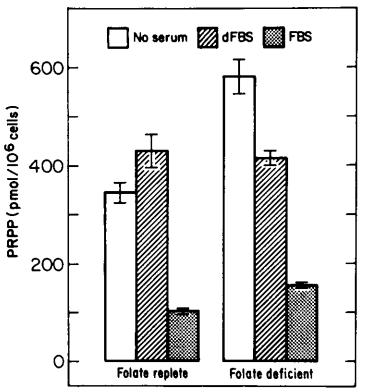

FIG. 3. Phosphate-induced PRPP elevations are abolished by the addition of PRPP-reacting substances in FBS but not dialyzed FBS (dFBS). The reaction mixture contains 20 μl FBS or dFBS and 20 m*M* PO_4.

Folate-dependent purine synthesis in HL-60 cells can be readily evaluated by the measurement of PRPP levels using a simplified, 2-hr incubation in the presence of PO_4 with and without folate (Fig. 5). In this two-time-point assay, the PRPP levels are indistinguishable in HL-60 cells grown in folate-replete media but remain markedly elevated in folate-deficient HL-60 cells. This folate deficiency-provoked persistent elevation of PRPP levels was corrected by the addition of either PteGlu or 5-CHO-H_4PteGlu and the PRPP level returned to that of the folate-replete cells (Fig. 5).

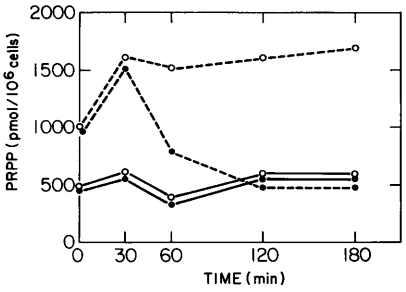

FIG. 4. Phosphate-induced elevations of PRPP are higher in folate-deficient HL-60 cells as compared to folate-replete cells. The addition of 5-CHO-H_4PteGlu (4 μM) to the incubation mixture lowers the PRPP levels of the folate-deficient cells to normal levels (folate-replete cells) within 2 hr. ○ --- ○, folate-deficient; ● --- ●, folate-deficient + 5-CHO-H_4PteGlu; ○ — ○, folate-replete; ● — ●, folate-replete + 5-CHO-H_4PteGlu.

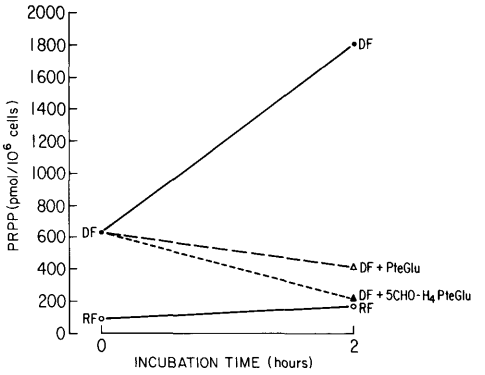

FIG. 5. PRPP levels in folate-replete (RF) and folate-deficient (DF) HL-60 cells before and after a 2-hr incubation with 20 mM PO_4 with or without PteGlu (100 μM) or 5-CHO-H_4PteGlu (4 μM).

The antipurine effect of antifolates such as MTX can be sensitively measured by assaying PRPP levels in the presence of high-molarity PO_4 in cells previously washed free of PRPP-reacting substances. As demonstrated in Fig. 6, when PRPP levels are measured following a 6-hr incubation with MTX without PO_4 added to the medium, it is difficult to demonstrate a dose–response curve for measuring MTX-induced elevation of PRPP levels. In contrast, when PO_4 was added to the tissue culture medium during the 6-hr MTX exposure, there was a sensitive dose–response curve of increasing PRPP levels starting at $<1 \times 10^{-8}$ M MTX. There is an increased sensitivity to MTX measurable at 2×10^{-9} M if the cells are washed free of PRPP-reacting substances in the tissue culture medium prior to the addition of PO_4 (data not shown). The deoxyuridine suppression assay (8) which is a sensitive method for measuring the MTX inhibition of *de novo* pyrimidine synthesis was studied under the same conditions. As shown in Table I, there was a similar MTX dose–response curve found using the deoxyuridine suppression of thymidine incorporation into DNA, which is first measurable at 10^{-8} M MTX and plateaus at 10^{-7} M MTX. In order to sensitively measure the rescue effect of 5-CHO-H_4PteGlu on MTX-induced elevations of PRPP in HL-60 cells it is also necessary to add PO_4 to the tissue culture medium (Table II). In this study with HL-60 cells incubated 3 hr with MTX together with 5-CHO-H_4PteGlu, it was not possible to demonstrate an effect on PRPP levels unless it was followed by an additional 3-hr incubation in the presence of PO_4. Under these conditions, there was a sixfold increase in the PRPP levels in the presence of MTX (10^{-7} M) which was decreased to normal levels when 5-CHO-H_4PteGlu (2×10^{-5} M) was added. However, 5-CHO-H_4PteGlu failed to lower PRPP levels in HL-60 cells previously incubated with MTX (10^{-7} M) for more than 3 hr (data not shown).

Since PO_4 was previously shown to stimulate PRPP synthetase in RBC (5) we reasoned that this mechanism could be utilized to load RBC with an antimetabolite such as 6-MP based upon a 1:1 stoichiometric reaction between the PO_4-induced elevation of intracellular PRPP and 6-MP catalyzed by hypoxanthine phosphoribosyltransferase. As shown in Fig. 7 there was a linear decrease in RBC PRPP levels induced by PO_4 following incubation

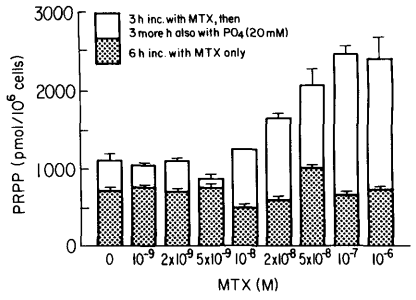

FIG. 6. HL-60 cells were incubated with MTX with and without added PO_4. Between 10^{-8} and 10^{-7} M MTX there is a progressive elevation of PRPP in cells incubated for 3 hr with MTX and a further 3 hr with 20 mM PO_4.

TABLE I. DEOXYURIDINE SUPPRESSION OF [^{3}H]THYMIDINE INCORPORATION INTO ACID-INSOLUBLE MATERIAL (DNA) OF HL-60 CELLS INCUBATED WITH INCREASING AMOUNTS OF MTX

Addition	Percentage of [^{3}H]thymidine[a] incorporation in the presence of 100 mM deoxyuridine (dU) (<10% = normal)
dU	5.0
dU + MTX (10^{-10} M)	4.1
dU + MTX (10^{-9} M)	3.1
dU + MTX (10^{-8} M)	37.7
dU + MTX (10^{-7} M)	42.0
dU + MTX (10^{-6} M)	46.0

[a] SA 20 Ci/mmole, 0.1 μCi/ml incubation.

TABLE II. PRPP LEVELS IN HL-60 CELLS INCUBATED WITH MTX, ALONE OR TOGETHER WITH 5-CHO-H_4PteGlu, FOLLOWED BY A FURTHER 3-hr INCUBATION WITH PO_4

Hours of incubation	MTX (10^{-7} M)	5-CHO-H_4PteGlu (2×10^{-5} M)	PO_4 (20 mM)	PRPP (pmole/10^6 cells)
0				400
3				640
6				480
3 + 3[a]			Yes	670
3	Yes			600
6	Yes			410
3	Yes	Yes		750
3 + 3[b]	Yes		Yes	2360
3 + 3[c]	Yes	Yes	Yes	560

[a] No additions for 3 hr, then PO_4 for 3 hr.
[b] MTX for 3 hr, then PO_4 for a further 3 hr.
[c] MTX + 5-CHO-H_4PteGlu for 3 hr, then PO_4 for a further 3 hr.

with increasing amounts of 6-MP. This is consistent with the utilization of PRPP by added 6-MP to generate and accumulate intracellular 6-MP nucleotide.

Discussion. The assay of PRPP levels appears to be a useful and sensitive method to assess the folate metabolic status of purine synthesizing cells. This has been demonstrated by an elevation of PRPP levels in several cells conditioned to folate deficiency by passage through culture medium containing 25 nM folate (4). The elevations in PRPP were directly related to a fall in intracellular folate levels (11). Incubation of folate-deficient cells with added 20 mM PO_4 makes the assay more sensitive by elevating PRPP levels still further.

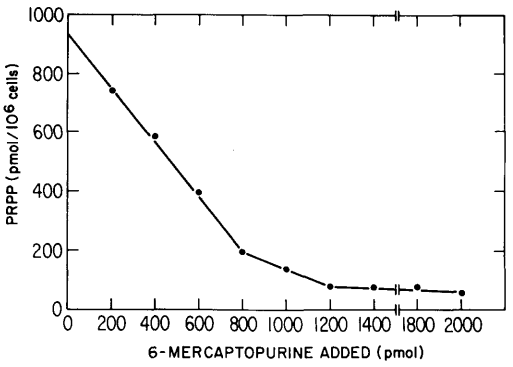

FIG. 7. 6-MP incorporation in RBC as measured by decreasing PRPP levels in RBC incubated for 60 min with 20 mM PO_4 and 0.5% glucose. The cells were washed with Tris–NaCl and then incubated with increasing amounts of 6-MP.

This is probably due to stimulation of PRPP synthesis by PO_4 combined with a rate-limiting role of PRPP utilization for purine synthesis due to folate deficiency. Thus, a rapid, 2-hr assay for assessing folate metabolism can be utilized by generating the PRPP level with PO_4 which remains elevated in folate-deficient cells unless excess folate is added to the incubation medium to allow for utilization of PRPP. In contrast, cells with normal intracellular folate levels and normal folate metabolism generate increased levels of PRPP in the presence of PO_4 that rapidly return to basal levels within 2 hr because of normal PRPP utilization for purine synthesis. This assay can be used for measuring the integrity of folate-dependent steps in purine synthesis and will be abnormal in nutritional folate deficiency. The assay was further adapted for this purpose by removing PRPP-reacting substances present in serum (i.e., hypoxanthine) which would interfere with the assay. Therefore, cells obtained for PRPP assay should be washed free of serum and then incubated in folate-free RPMI 1640 plus 15% dialyzed FBS containing 20 mM PO_4.

Assaying PRPP in cultured cells stimulated by added PO_4 is also useful in monitoring inhibitors of purine metabolism, such as MTX and 6-methyl mercaptopurine riboside (6-MMPR). MTX-induced elevations of PRPP utilizing PO_4 stimulation can be detected at levels as low as 2×10^{-9} M. In contrast, prior reports in human colorectal adenocarcinoma HCT-8 cells not utilizing PO_4 in the PRPP assay required 1×10^{-5} M MTX to provoke PRPP elevations (12). The present assay is as

sensitive for measuring derangement of purine synthesis induced by MTX as the deoxyuridine suppression assay is for measuring derangements in pyrimidine synthesis as a result of MTX. The elevations in PRPP induced by MTX can be reversed if 5-CHO-H_4PteGlu is given within 3 hr after MTX exposure but fails to decrease PRPP levels significantly thereafter over a 12-hr observation. Therefore, it would appear that in some cells the increased levels of PRPP induced by low levels ($<10^{-7}$ M) of MTX can be used for modulation of 5-fluorouracil (5-FU) and maintained for long periods of time if 5-CHO-H_4PteGlu is given more than 3 hr after MTX and prior to 5-FU. This should allow for a more effective biochemical modulation of 5-FU by increasing the amount of PRPP and intracellular folate and by the subsequent binding of 5-FU nucleotide to thymidylate synthetase, the target enzyme (13, 14).

PO_4 stimulation of PRPP in intact RBC may be useful for delivery of chemotherapeutic agents to the reticuloendothelial system. We have demonstrated that PO_4 stimulates the incorporation of 6-MP in RBC. This procedure could be useful for targeting sensitized RBC loaded with 6-MP to the reticuloendothelial system for the treatment of certain immune disorders such as autoimmune hemolytic anemia. Thus, assaying PRPP in fresh cells or from tissue culture stimulated by added PO_4 is useful in monitoring their folate-dependent purine metabolic status and their response to pertinent antimetabolites, and for incorporating antimetabolites that utilize PRPP into cells to target chemotherapy.

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