Role of Cellular Ribose-5-Phosphate Content in the Regulation of 5-Phosphoribosyl-1-Pyrophosphate and De Novo Purine Synthesis in a Human Hepatoma Cell Line

Pnina Boer and Oded Sperling

5-Phosphoribosyl-1-pyrophosphate (PRPP) is an important regulator of de novo purine synthesis. However, the role of ribose-5-phosphate (R5P), the precursor for PRPP, in the regulation of PRPP and de novo purine synthesis has not yet been clarified conclusively. This study was designed to clarify interrelationships between R5P content, PRPP availability, and the rate of de novo purine synthesis in the cultured human hepatoma cell line ($HepG_2$), a plausible model for normal human hepatocytes. Increasing glucose concentration in the culture media from 0 to 10 mmol/L resulted in a 2.9-fold elevation of cellular R5P content (from 107 ± 31 to 311 ± 57 nmol/g protein), associated with a correlated increase of 7.14-fold in cellular PRPP availability (from 4.76 ± 3.4 to 34 ± 8.4 pmol/mg protein/min) and of 149-fold in the rate of de novo purine synthesis (from 55 to 8,204 dpm/mg protein/h). Plotting the rate of de novo purine synthesis versus R5P content indicates that at a wide range of R5P content, including that prevailing in hepatocytes under physiological conditions, the rate of purine synthesis depends on R5P content. A similar dependence was also demonstrated for PRPP availability. The rate of de novo purine synthesis exhibited a sigmoidal dependence on PRPP availability. The demonstration in human hepatocytes of dependence of the rate of purine synthesis on R5P content has implications concerning the pathogenesis of purine overproduction associated with several inborn and acquired conditions in man.

AN IMPORTANT REGULATOR of de novo purine synthesis is 5-phosphoribosyl-1-pyrophosphate (PRPP).[1-4] It is both a substrate and an activator of the phosphoribosyl pyrophosphate amidotransferase (EC 2.4.2.14), catalyzing the first-committed and rate-limiting reaction in the pathway of de novo purine synthesis.[5] In intact cells, increased availability of PRPP correlates with enhanced de novo purine synthesis,[6-8] whereas decreased availability of PRPP is associated with decelerated purine synthesis.[9-11] The availability of ribose-5-phosphate (R5P), the precursor of PRPP, has also been suggested to regulate purine synthesis, through modulation of PRPP availability.[1-3,12] Moreover, increased availability of R5P due to various defects affecting carbohydrate metabolism was suggested to be the link between the primary defect and excessive purine synthesis in several conditions, including glycogen storage disease type I[13] and glutathione reductase superactivity associated with gout.[14]

Nevertheless, whether R5P availability may indeed play a role in the regulation of purine synthesis in man depends on conclusive evidence for such a possibility in human liver tissue. The liver has a major role in both anabolism and catabolism of purine nucleotides in the body, and excessive purine synthesis is probably a reflection of liver metabolism. That R5P availability may indeed regulate liver purine metabolism is indicated by the results of a study in rats in vivo.[15] In man, a regulatory role for R5P in PRPP and in de novo purine synthesis was demonstrated in cultured human lymphoblasts,[16] but not yet in liver tissue.

In the present study, we chose to clarify the relationship between R5P content, PRPP availability, and the rate of de novo purine synthesis in a human hepatoma cell line ($HepG_2$), a widely used in vitro model for human hepatocytes.[17,18] Cell content of R5P was modulated by varying glucose concentration in the culture media. In addition, the effect of various substances anticipated to affect cellular R5P content was studied. The results demonstrate that in the human hepatocyte line under physiological concentrations of glucose and inorganic phosphate, R5P availability is rate-limiting for PRPP and de novo purine synthesis.

MATERIALS AND METHODS

Materials

Ribose, pyrroline-5-carboxylate, phenazine methosulfate, methylene blue, epinephrine, dibutyryl cyclic adenosine monophosphate (cAMP), PRPP synthetase (EC 2.7.6.1; from *Escherichia coli*), hypoxanthine-guanine phosphoribosyltransferase (EC 2.4.2.8; from baker's yeast), HEPES buffer, and all other unspecified fine chemicals were obtained from Sigma (St Louis, MO). Insulin was prepared by Lilly (Giessen, Germany). (8-^{14}C)hypoxanthine (50 to 62 mCi/mmol), (8-^{14}C)adenine (50 to 62 mCi/mmol), and (^{14}C)formate (sodium salt, 50 to 62 mCi/mmol) were obtained from Amersham Radiochemical Center (Buckinghamshire, UK). Dulbecco's modified Eagle's medium with low glucose, deficient Dulbecco's modified Eagle's medium (without glucose, glutamine, and sodium pyruvate), fetal calf serum, and glutamine were obtained from Sigma and from Biological Industries (Kibbutz Beth Haemek, Israel). Culture dishes were obtained from Corning Glass Works (Corning, NY).

Cells

$HepG_2$ cells were cultured in Dulbecco's modified Eagle's medium with low glucose (1,000 mg/L) supplemented with fetal calf serum (15%), glutamine (2 mmol/L), penicillin (100 U/mL), streptomycin (0.1 mg/mL), and nystatin (25 U/mL). Cells were

From the Felsenstein Medical Research Center and the Department of Clinical Biochemistry, Beilinson Medical Center, Petah-Tikva; and the Department of Clinical Biochemistry, Sackler Faculty of Medicine, Tel Aviv University, Tel Aviv, Israel.

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Address reprint requests to Oded Sperling, PhD, Department of Clinical Biochemistry, Beilinson Medical Center, Petah-Tikva 49100, Israel.

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grown in 25-cm^2 plastic tissue culture flasks in a humidified incubator at 37°C under an atmosphere of 5% CO_2/95% air and passaged once per week. All experiments were performed in this medium, except for experiments in which glucose concentration was modified. In such experiments, the regular culture medium was changed 24 hours before experiments to deficient Dulbecco's modified Eagle's medium (without glucose, glutamine, and sodium pyruvate) without serum, adjusted to contain various concentrations of glucose (0 to 10 mmol/L), or to Earle's balanced salt solution modified to contain various glucose concentrations (0 to 10 mmol/L) and HEPES buffer, pH 7.4 (20 mmol/L). The various effectors were added at the time and in the concentration specified.

Analytical Procedures

R5P concentration. R5P was determined by a coupled enzymatic assay. R5P was converted to PRPP, which was reacted with labeled hypoxanthine to yield labeled inosine monophosphate.[12] Cells from two confluent 25-cm^2 flasks were washed two times with ice-cold NaCl 0.9%, scraped off, and transferred to a conical glass tube. The cell suspension in NaCl 0.9% was centrifuged at 4,000 rpm for 10 minutes at 4°C. Twenty-five microliters of cold water was added to the cell pellet, and the cells were lysed by freezing and thawing four times in a CO_2-acetone bath. The frozen cell lysates were kept until extraction in the CO_2-acetone bath. For extraction of R5P, cell lysates were thawed slightly by keeping the tube in ice for 1 minute and then transferred with the aid of a spatula into the bottom of a preheated (100°C for 5 minutes) Corex tube containing 250 μL 20-mmol/L Tris hydrochloride buffer, pH 7.4, and 50 μmol/L (8-^{14}C)hypoxanthine (50,000 dpm/nmol). The contents of the tube were mixed quickly, and the tube was put back in the boiling-water bath for 20 seconds and cooled immediately on ice. Following centrifugation, 200 μL of the supernatant (R5P extract) was taken for R5P determination. The R5P assay mixture, a total volume of 240 μL, contained 0.5 mmol/L adenosine triphosphate (ATP), 5 mmol/L $MgCl_2$, 2.5 mmol/L mercaptoethanol, 10 mmol/L K_2HP_4, 33 U/L PRPP synthetase, and 833 U/L hypoxanthine-guanine phosphoribosyltransferase. The reaction was continued to completion (1 hour at 37°C). The proteins were precipitated by addition of perchloric acid to a concentration of 0.5 mol/L followed by centrifugation. The (^{14}C)inosine monophosphate product was separated from the substrate (^{14}C)hypoxanthine by thin-layer chromatography on microcrystalline cellulose plates using *n*-butanol:methanol:water:25% NH_4OH (60:20:20:1 vol/vol) as solvent. Recovery of a R5P standard subjected to this extraction by a heating procedure was 96% to 100%. The method was linear up to at least 15 μmol/L. This assay procedure determines both PRPP and R5P. Nevertheless, in view of the fact that PRPP content in comparison to that of R5P in the cell extracts is negligible (<5%; PRPP was measured by the same assay system, omitting ATP and PRPP synthetase), and due to the unsatisfactory accuracy of PRPP determination, PRPP content of the extracts was not assayed.

The effect of the various substances on R5P content was studied following incubation of the cultures with the effectors for 1 hour.

Protein level was measured according to the method reported by Lowry et al.[19]

PRPP availability. The metabolic availability of PRPP was gauged by measuring the rate of (8-^{14}C)adenine incorporation into the intact-cell total nucleotide pool. $HepG_2$ cells were plated on 35-mm plastic tissue culture dishes and cultured for 3 days, following which the amount of cells in each dish was comparable to 0.5 to 1.0 mg protein. For assay of PRPP availability, the medium was substituted by 1 mL fresh medium containing (8-^{14}C)adenine (5 μmol/L; 45,000 dpm/nmol), and the cells were incubated for 15 minutes. Following incubation, the medium was discarded and cells were washed two times with ice-cold NaCl 0.9% (2 mL). Total acid-soluble purines were extracted from the hepatocytes by addition of 250 μL ice-cold perchloric acid 0.5 mol/L, followed by mechanical scraping of the cells from the dish. Following centrifugation (at 4°C), the labeled nucleotides were separated from the substrate (^{14}C)adenine by thin-layer chromatography on microcrystalline cellulose using *n*-butanol:methanol:water:25% NH_4OH (60:20:20:1 vol/vol) as solvent. Under the conditions specified earlier, incorporation of (^{14}C)adenine was linear for at least 30 minutes.

For the study of the effect of glucose concentration, the regular medium was substituted 24 hours before the experiment with modified Earle's balanced salt solution containing the required glucose concentration. The same glucose concentration was included in the (8-^{14}C)adenine-containing incubation medium. For the study of the effect of various other substances on PRPP availability, the cultures were preincubated with the effectors for 15 to 60 minutes (as specified) before addition of the (8-^{14}C)adenine-containing medium.

De novo purine synthesis. The rate of de novo purine synthesis was gauged by the rate of (^{14}C)formate incorporation into the acid-soluble cellular purines and into purines excreted by the cells into the incubation medium.[20] Cells were grown for 3 days before the assay in 35-mm dishes under regular conditions. The culture medium was discarded and substituted by 1 mL fresh medium containing (^{14}C)formate (43 μmol/L; 2.15 μCi per dish). Incubation was performed for 1 hour. After incubation, the culture medium was separated from the cells and purine nucleotides, and nucleosides in the cells and in the medium (separately) were hydrolyzed (at 1 mol/L perchloric acid at 100°C for 1 hour). The purine bases were precipitated as silver salts and counted as described previously.[20] Under these conditions, $HepG_2$ cells were found to incorporate (^{14}C)formate linearly for at least 2 hours, and at 1 hour the rate of incorporation increased with formate concentration up to at least 86 μmol/L. Five percent to 10% of the labeled purines were excreted into the medium (Fig 1). Incorporation of (^{14}C)formate into purines was inhibited completely by adenine (1 mmol/L) and 87% by hypoxanthine (1 mmol/L). The presence of 10% fetal calf serum in the medium inhibited incorporation by 50% (the serum contains purine bases and nucleosides, which are known to inhibit purine synthesis de novo).

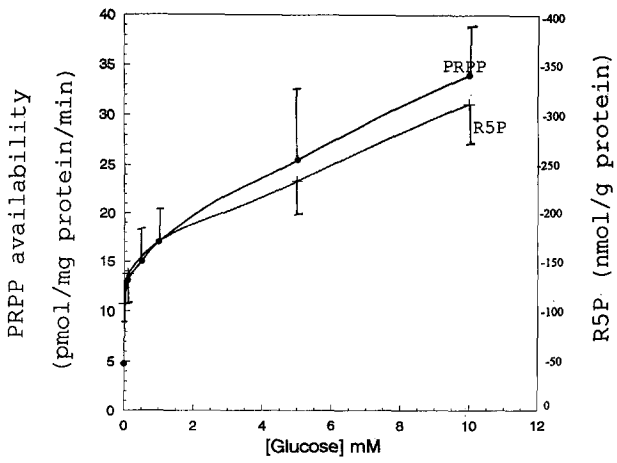

Fig 1. Effect of glucose on R5P content and on PRPP availability in $HepG_2$ cells. The culture medium was changed to modified Earle's balanced salt solution containing 0 to 10 mmol/L glucose, and incubation continued for 24 hours, following which R5P content and PRPP availability were determined. Mean ± SD of 6 to 10 independent determinations.

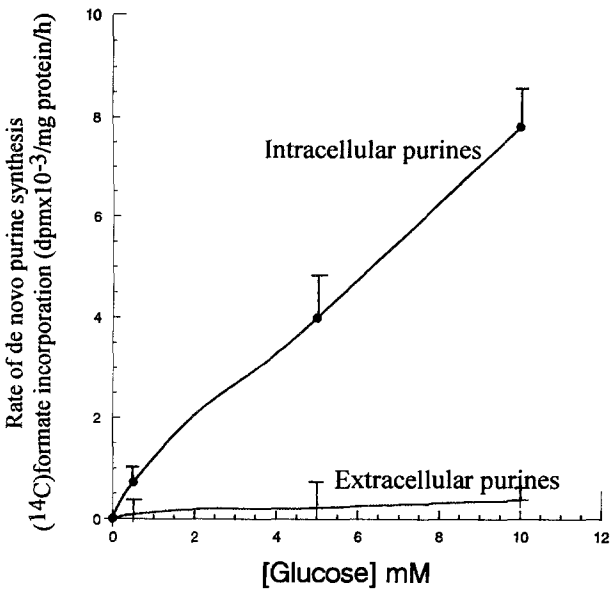

Fig 2. Effect of glucose concentration on the rate of de novo purine synthesis in cultured $HepG_2$ cells. The culture medium was changed, 24 hours before determination of the rate of (^{14}C)formate incorporation, to glucose-deficient Dulbecco's modified Eagle's medium (without serum) adjusted to contain 0 to 10 mmol/L glucose. Mean ± SD of 4 to 6 independent determinations.

RESULTS

At the physiological glucose concentration of 1,000 mg/L, R5P content was 233 ± 42 nmol/g protein (13 determinations), PRPP content was 8.39 ± 42 nmol/g protein (37 determinations), and PRPP availability was 25 ± 9.8 pmol/mg protein/min (12 determinations) (Fig 1).

R5P content and the metabolic availability of PRPP in $HepG_2$ cells exhibited dependence on glucose concentration. Following incubation of $HepG_2$ cultures for 24 hours in Earle's salt solution containing 0 to 10 mmol/L glucose, R5P content increased from 107 ± 31 nmol/g protein at 0 mmol/L glucose to 311 ± 57 nmol/g protein at 10 mmol/L glucose. PRPP availability increased similarly from 4.76 ± 3.4 pmol/mg protein/min at 0 mmol/L glucose up to 34 ± 8.4 pmol/mg protein/min at 10 mmol/L glucose (Fig 1). Values for R5P content and PRPP availability similar to those obtained following incubation in Earle's salt solution in relation to glucose concentration were also obtained following incubation of the cultures in Dulbecco's modified Eagle's medium.

The rate of (^{14}C)formate incorporation, gauging the rate of de novo purine nucleotide synthesis, was studied in Dulbecco's modified Eagle's medium without serum. In this medium, the rate of incorporation into purines exhibited an almost linear dependence on glucose concentration, ranging from 55 dpm/mg protein/h at 0 mmol/L glucose up to 8,204 dpm/mg protein/h at 10 mmol/L glucose (Fig 2).

Several potential accelerators of R5P generation were studied for their effect on cellular R5P content, PRPP availability, and rate of de novo purine synthesis. The activators of the oxidative pentose phosphate pathway, phenazine methosulfate, pyrroline-5-carboxylate, and methylene blue, were found to increase R5P content significantly by sevenfold, 2.5-fold, and 1.7-fold, respectively. All other compounds, including sodium acetate, ethanol, and inorganic phosphate, which were reported to increase R5P content in rat liver in vivo,[21] as well as dibutyryl cAMP and epinephrine, did not increase cellular R5P content in cultured hepatocytes (Table 1).

The effect of accelerators of the oxidative pentose phosphate pathway on PRPP availability was studied at physiological (1 mmol/L) and high (10 mmol/L) inorganic phosphate concentrations, at which PRPP synthetase is activated.[22] Exposure to phenazine methosulfate (0.1 mmol/L for 1 hour) resulted in an elevation of PRPP availability of 70% at 1 mmol/L inorganic phosphate and 85% to 130% at 10 mmol/L phosphate, but only at low, nonphysiological glucose concentrations (<0.5 mmol/L glucose). At higher glucose concentrations, addition of phenazine methosulfate caused a decrease in PRPP availability. The two other accelerators of the oxidative pentose phosphate pathway, methylene blue (added at 0.1 mmol/L for 1 hour) and pyrroline-5-carboxylate (at 0.1, 0.5, and 1.5 mmol/L for 1 hour), decreased PRPP availability at all glucose concentrations studied (0, 0.1, and 5 mmol/L).

Incubation of the cultures with ribose (0.05 to 1 mmol/L for 15 minutes) following glucose starvation for 24 hours did not elevate PRPP availability and did not affect the rate of de novo purine synthesis.

The effect of the glycogenolytic hormones epinephrine and glucagon, of dibutyryl cAMP, and of insulin on PRPP availability and on the rate of de novo purine synthesis was studied in $HepG_2$ cell cultures, at concentrations earlier demonstrated to increase tissue content of PRPP and the rate of de novo purine synthesis in mouse liver in vivo and in rat hepatocytes in suspension.[23-25] Incubation of the cultures for 45 minutes with epinephrine (10^{-4} mol/L), glucagon (10^{-6} mol/L), dibutyryl cAMP (10^{-3} mol/L), and insulin (5 to 100 U/L) at 0, 1, and 10 mmol/L glucose concentrations did not increase PRPP availability and did not enhance the rate of de novo purine synthesis.

Table 1. Effect of Different Potential Accelerators of R5P Generation on R5P Content in $HepG_2$ Cells

Substance	R5P (nmol/g protein)
—	233 ± 42 (13)
Pyrroline-5-carboxylate (1.5 mmol/L)	591 ± 151 (3)
Phenazine methosulfate (0.1 mmol/L)	1,683 ± 131 (3)
Methylene blue (0.1 mmol/L)	409 ± 65 (3)
Sodium acetate (20 mmol/L)	188 ± 30 (3)
Ethanol (70 mmol/L)	181 ± 21 (3)
K_2HPO_4 (10 mmol/L)	203 ± 21 (3)
Dibutyryl cAMP (1 mmol/L)	216 ± 22 (3)
Epinephrine (100 μmol/L)	164 ± 23 (3)

NOTE. The regular culture medium (Dulbecco's modified Eagle's medium containing 1,000 mg/L glucose) was changed to a fresh medium containing the various materials. Cells were then incubated for 1 hour, and R5P was extracted and determined. Each value represents the mean ± SD of at least 3 independent determinations (number of determinations is indicated in parentheses).

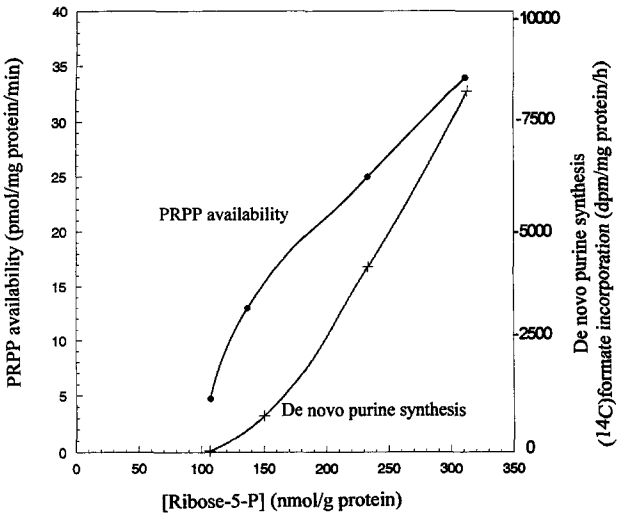

Fig 3. Relationship between R5P content and PRPP availability and between R5P content and the rate of de novo purine synthesis in cultured $HepG_2$ cells. PRPP availability and the rate of de novo purine synthesis in cells were plotted versus concentrations of R5P. Values were obtained from results depicted in Figs 1 and 2.

DISCUSSION

The results indicate clearly that in the intact cultured $HepG_2$ cell line, representing human liver tissue, rates of PRPP generation and de novo purine synthesis depend on R5P availability. Modification of glucose concentration in the culture medium resulted in correlated alterations in cellular R5P content, PRPP availability, and rate of purine synthesis (Figs 1 and 2). The relationships in intact hepatocytes between R5P content and PRPP availability, between R5P content and rate of de novo purine synthesis, and between PRPP availability and rate of de novo purine synthesis were calculated from the results presented in Figs 1 and 2, and are presented in Figs 3 and 4. At the concentration range of R5P in hepatocytes from 100 to 320 nmol/g protein (obtained at 0 to 10 mmol/L glucose in the incubation media), PRPP availability and the rate of de novo purine synthesis exhibit a strong dependence on R5P concentration (Fig 3).

The results of this study are compatible with those of several other investigations, demonstrating or suggesting a role for R5P in the regulation of PRPP and purine synthesis. A positive correlation between R5P and PRPP was found in hepatic tissue of rats treated by different diets and under various hormonal states.[15] A regulatory role for R5P in PRPP and in de novo purine synthesis was demonstrated in cultured human lymphoblasts.[16]

The demonstration in this cultured hepatocyte line of the dependence of PRPP generation on the availability of R5P (Fig 3) is also compatible with the reported values of the K_m for PRPP synthetase (EC 2.7.6.1) for R5P in relation to the tissue concentration of R5P. In several studies, the K_m of PRPP synthetase for R5P was found to be 3.3×10^{-5} mol/L (for human red blood cells[26]), 3 to 5×10^{-5} mol/L (for human lymphoblasts[27]), and 2.9×10^{-4} mol/L (for rat liver enzyme[28]). The concentration of R5P was found to be 3.5 and 8 μmol/L in rat liver[29,30] and 137 μmol/L in human lymphoblasts.[27] It should be emphasized that these K_m values were obtained at high, nonphysiological concentrations of inorganic phosphate (40 to 50 mmol/L), activating PRPP synthetase. At lower physiological concentrations of phosphate (1 to 5 mmol/L), K_m values are probably higher.[27]

The observation in the human hepatoma cell line of the dependence of the rate of de novo purine synthesis on R5P availability furnishes a possible mechanism by which perturbations in carbohydrate metabolism associated with increased availability of R5P can lead to acceleration of purine synthesis. Increased R5P availability has indeed been suggested to be the reason for purine overproduction in glucose-6-phosphatase deficiency (glycogen storage disease type I, ie, Gierke's disease[13]). However, more recent studies suggest that accelerated ATP breakdown is the primary defect leading to purine overproduction in this inborn error of metabolism.[31,32] Increased R5P generation was also suggested to underlie purine overproduction in gouty subjects affected with superactivity of glutathione reductase.[14] Increased availability of R5P should be investigated in other purine-overproducing patients in whom the underlying metabolic defect is still unidentified. Indeed, an increased R5P concentration was found in cultured fibroblasts derived from two purine-overproducing patients.[12]

Plotting the relationship between the rate of de novo purine synthesis and PRPP availability in the intact hepatocyte showed a sigmoidal dependence of the latter on the former (Fig 4). The sigmoidal response of the phosphoribosyl pyrophosphate amidotransferase to increasing PRPP availability in the intracellular milieu, containing purine nucleotide inhibitors, indicates that PRPP acts as an allosteric activator of the amidotransferase.[4] In the human

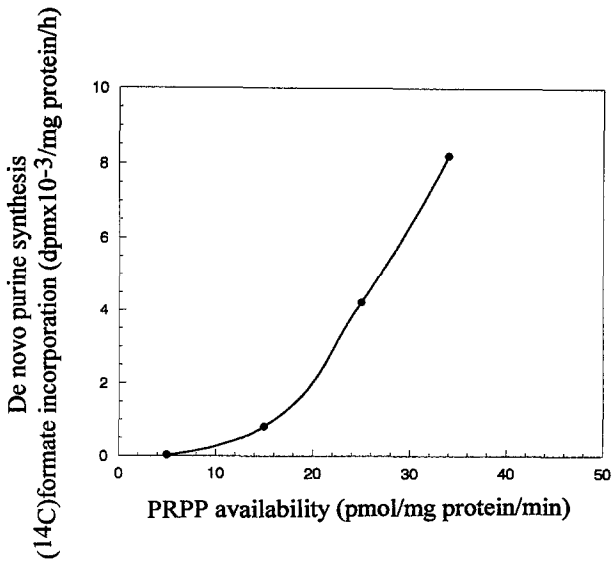

Fig 4. Relationship between PRPP availability and the rate of de novo purine synthesis in cultured $HepG_2$ cells. The rate of de novo purine synthesis was plotted versus the availability of PRPP. Values were obtained from results depicted in Figs 1 and 2.

hepatocyte line studied, the maximal velocity of de novo purine synthesis was observed at the range of PRPP availability obtained at physiological glucose concentrations (4.5 to 6.5 mmol/L). Thus, the rate of de novo purine nucleotide synthesis is most sensitive to PRPP availability at the physiological range of PRPP. This first demonstration of the interrelationship between PRPP availability and the rate of de novo purine synthesis in human hepatocytes is in accordance with the established role of PRPP availability in the regulation of the rate of purine synthesis in man. The best example for this role is the excessive purine production associated with the mutations of PRPP synthetase superactivity[20] and of hypoxanthine-guanine phosphoribosyltransferase deficiency.[33]

In view of the demonstrated effect of glucose on R5P content, on PRPP availability, and on the rate of de novo purine synthesis, we studied the effect of several additional compounds reported to increase R5P generation in intact cells and tissues. Three accelerators of the oxidative pentose phosphate pathway, acting via oxidation of NADPH to NADP, were studied: pyrroline-5-carboxylate, phenazine methosulfate, and methylene blue. These three effectors were indeed found to enhance R5P generation in the $HepG_2$ cell line, but they failed to accelerate PRPP production. This finding is in contrast to the conclusive evidence demonstrated in this study for the role of R5P availability in the regulation of PRPP synthesis. However, it should be emphasized that previous attempts to enhance PRPP production by these substances in various cells and tissues, as well as in whole animals,[6-8,12,34-37] produced contradictory results. No clear explanation is available for the lack of effect of these substances on PRPP availability in the present study, as well as in other studies. Perhaps the main pathway generating R5P for PRPP production is the nonoxidative rather than the oxidative direction of the pentose phosphate pathway.[38] The lack of effect or even the inhibitory effect of accelerators of the oxidative pathway on PRPP generation observed in the present study at certain conditions may also be attributed to a direct inhibitory effect of these substances or metabolites produced as a result of their effect on PRPP synthetase. In addition, it could reflect inhibition of PRPP synthetase by the high concentrations of substrate R5P generated by the electron acceptors. Substrate inhibition of purified rat liver PRPP synthetase by R5P concentrations above 1.5 mmol/L was demonstrated by Roth et al.[28]

The lack of effect of ribose on PRPP availability and on the rate of de novo purine synthesis can be explained by the absence of ribokinase in $HepG_2$ cells, as in other cells,[16] but this possibility has not yet been verified.

In contrast to previous findings in mice in vivo[23,24] and in fresh rat hepatocyte suspensions,[25,37] the glycogenolytic hormones, dibutyryl cAMP, and insulin did not increase PRPP availability and did not enhance the rate of de novo purine synthesis in $HepG_2$ cells. It is possible that some changes occurred in the $HepG_2$ cell line in comparison to fresh tissue, abolishing the effects of the hormones and of dibutyryl cAMP. The nature of these changes has not yet been clarified.

In contrast to the findings reported by Kim et al[21] in rat liver, no increase in R5P content could be detected in $HepG_2$ cells under the influence of ethanol, its metabolic product acetate, or inorganic phosphate.

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