Hemolytic Anemia in Hereditary Pyrimidine 5'-Nucleotidase Deficiency: Nucleotide Inhibition of G6PD and the Pentose Phosphate Shunt

By Akio Tomoda, Nancy A. Noble, Neil A. Lachant, and Kouichi R. Tanaka

We evaluated the erythrocytes of two patients with hereditary pyrimidine 5'-nucleotidase deficiency. Significant findings included an increased reduced glutathione content, increased incubated Heinz body formation, a positive ascorbate cyanide test, and decreased intraerythrocytic pH. The pentose phosphate shunt activity of the patients' red cells as measured by the release of ^14CO_2 from ^14C-1-glucose was decreased compared to high reticulocyte controls. Glucose-6-phosphate dehydrogenase (G6PD) activity in hemolysates from control erythrocytes was inhibited 43% by 5.5 mM cytidine 5'-triphosphate (CTP) and 50% by 5.5 mM uridine 5'-triphosphate (UTP) at pH 7.1. CTP was a competitive inhibitor for G6P (K_I = 1.7 mM) and a noncompetitive inhibitor for NADP (K_I = 7.8 mM). Glutathione peroxidase, glutathione reductase, and 6-phosphogluconate dehydrogenase were not affected by these compounds. Pentose phosphate shunt activity in control red cell hemolysate at pH 7.1 was inhibited to a similar degree by 5.5 mM CTP or UTP. Since the intracellular concentrations of G6P and NADP are below their K_I's for G6PD, these data suggest that high concentrations of pyrimidine 5'-nucleotides depress pentose phosphate shunt activity in pyrimidine 5'-nucleotidase deficiency. Thus, this impairment of the pentose phosphate pathway appears to contribute to the pathogenesis of hemolysis in pyrimidine 5'-nucleotidase deficiency hemolytic anemia.

ERYTHROCYTE PYRIMIDINE 5'-nucleotidase deficiency is characterized clinically by a chronic nonspherocytic hemolytic anemia and splenomegaly. In their initial description of pyrimidine 5'-nucleotidase deficiency, Valentine et al. demonstrated that these erythrocytes contain increased concentrations of pyrimidine 5'-nucleotides and increased reduced glutathione. In his preliminary investigations, Valentine observed an increase in Heinz body formation after incubating pyrimidine 5'-nucleotidase deficient red cells with acetylphenylhydrazine despite "normal" pentose phosphate shunt activity. Buc et al. observed that unstimulated pentose shunt activity was similar to that seen in normal reticulocyte controls. The activities of G6PD, 6PGD, glutathione reductase, and glutathione peroxidase have been shown to be normal in hemolysates from pyrimidine 5'-nucleotidase deficient erythrocytes. Thus, the mechanisms for the chronic hemolysis and the increased tendency to Heinz body formation have remained unclear.

This report presents pentose phosphate shunt and metabolic data on erythrocytes from two patients with pyrimidine 5'-nucleotidase deficiency and studies on the effect of pyrimidine nucleotides on enzyme kinetics.

CASE PRESENTATION

Case 1

Case 1 is a 46-yr-old white woman with previously reported pyrimidine 5'-nucleotidase deficiency hemolytic anemia. Significant past history included a cholecystectomy (gallstones present) and splenectomy at age 12, and left mastectomy for breast carcinoma at age 39. Her current hemogram includes: packed cell volume (PCV) 36%, hemoglobin 12.4 g/dl, red cell count 2.82 x 10^12/l, mean corpuscular volume (MCV) 106 fl, and 19.2% reticulocytes. The peripheral blood smear shows coarse basophilic stippling, Howell-Jolly bodies, and Pappenheimer bodies. The patient's pyrimidine 5'-nucleotidase activity (µmol - gHb^-1 - hr^-1) was decreased with both cytidine 5'-monophosphate (CMP) and uridine 5'-monophosphate (UMP) as substrates, while that of her heterozygous mother was intermediate between patient and control (patient 1.96 and 1.78, mother 3.59 and 8.73, and control 8.20 and 13.6, respectively).

Case 2

Case 2 is a 2.5-yr-old white girl whose past history is unremarkable except for chronic hemolytic anemia secondary to pyrimidine 5'-nucleotidase deficiency diagnosed at 16 mo of age. Palpable splenomegaly was not present at that time. Her most recent hemogram includes: PCV 32%, hemoglobin 9.8 g/dl, red cell count 2.81 x 10^12/l, MCV 112 fl, and 14.0% reticulocytes. Pyrimidine 5'-nucleotidase activity (µmol - gHb^-1 - hr^-1) with CMP and UMP as substrates were: patient 1.35 and 0.68, mother 3.34 and 3.86, and control 7.10 and 6.05, respectively.

MATERIALS AND METHODS

Patients

After obtaining informed consent, venous blood samples were collected with heparin as anticoagulant from the two patients, their heterozygous mothers, normal volunteers, and individuals with high reticulocyte nonenzymatic hemolytic anemia (sickle cell disease, immune hemolytic anemia, α-thalassemia).

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Materials

All reagents were purchased from Sigma Chemical Co. (St. Louis, MO.), except for tert-butyl-hydroperoxide (ICN Pharmaceuticals, Inc., Plainview, N.Y.) and \(^{4}\)C-1-glucose (New England Nuclear, Boston, Mass.). Solutions of the pyrimidine 5'-nucleotides, nicotinic adenine dinucleotide phosphate (NADP), adenosine triphosphate (ATP) and Tris-HCl were freshly prepared and neutralized with 1 N NaOH before use.

General Red Cell Methods

White cell and platelet-free red cell suspensions were prepared using \(\alpha\)-cellulose and microcrystalline cellulose according to Beutler and adjusted to red cell counts of between 2.8 and 3.2 \(\times\) 10\(^6\) RBC/\(\mu\)l.\(^8\)

Pyrimidine 5'-nucleotidase activity was determined according to Valentine et al.\(^1\) The methods used in this laboratory for determining glycolytic enzyme, G6PD, 6PGD, glutathione reductase and glutathione peroxidase, transaldolase, and transketolase activities have been previously published.\(^9\) The ascorbate cyanide test was performed according to the method of Jacob and Jandl.\(^16\) Heinz body formation was determined by a modification of the method of Beutler.\(^11\) Of whole blood, 0.1 ml (PCV adjusted to 40%) was incubated for 4 hr in 2 ml of 0.066 M phosphate buffer with 1 mg/ml acetylphenylhydrazine and 30 mg/dl glucose. Heinz bodies were detected by staining with crystal violet. Whole blood and red cell intracellular pH were measured by a modification of the method of Hilpert\(^2\) using microhematocrit tubes and a Radiometer micro-pH electrode.

Pentose Phosphate Shunt Activity

Pentose phosphate shunt activity was determined by a modification of the method of Davidson and Tanaka.\(^13\) The release of \(^{14}\)CO\(_2\) from \(^{14}\)C-1-glucose was measured in a vibrating reed electrometer and ionization chamber. The original method has been modified so that the build-up of \(^{14}\)CO\(_2\) is measured in a closed system. Fifty microliters of packed red cells were suspended in 1 ml of pH 7.4 Krebs-Ringer bicarbonate buffer. After 1 hr, the system was stimulated with 10 \(\mu\)l of 10\(^{-4}\) M new methylene blue (Sigma B-4631). \(^{14}\)CO\(_2\) production was continuously monitored for a total of 2 hr. Pentose phosphate shunt activity in red cell hemolysates was determined by a modification of the method of Smith.\(^14\) Krebs-Ringer bicarbonate buffer was supplemented with 1 mM ATP and 2 mM NADP. Fifty microliters of red cell hemolysate (1.5 dilution of packed red cells with distilled water) were added to 400 \(\mu\)l of buffer. New methylene blue was not added to the system.

Effects of Pyrimidine 5'-Nucleotides on Red Cell Enzymes

White cell and platelet-free red cells obtained from normal volunteers were washed 3 times with isotonic NaCl solution and then lysed with 20 vol of ice-cold distilled water. The red cell membranes were removed by centrifugation at 4\(^\circ\)C for 20 min at 10,000 g. The supernatant was used for the measurement of G6PD, 6PGD, glutathione reductase (GR), and glutathione peroxidase (GP) activities. All assays were performed at 37\(^\circ\)C. The detailed conditions for G6PD and 6PGD activity measurements are described under the legends to the figures. The hemoglobin concentrations were measured by the cyanmethemoglobin method.

Statistics

Data are given as mean \(\pm\) 1 standard derivation. Student's \(t\) tests were performed by standard statistical methods.\(^15\)

RESULTS

RBC Reduced Glutathione and Enzyme Activity

The reduced glutathione (GSH) content of the patients' red cells was elevated compared to the normal controls. GSH values (\(\mu\)g/10\(^6\) RBC) were: case 1, 776 (as high as 1150 previously); case 2, 959; and control, 607 \(\pm\) 79. The activity of the enzymes of the Embden-Meyerhof pathway, G6PD, 6PGD, glutathione reductase, glutathione peroxidase, transketolase, and transaldolase was normal for both patients and their mothers.

Heinz Body Formation, Ascorbate Cyanide Test, and Pentose Phosphate Shunt Activity

Incubated Heinz body formation was significantly increased in the patients' (36% and 24%) red cells compared to their mothers (10% and 19%) and the normal controls (11% \(\pm\) 5%, \(p < 0.005\)). Both patients' red cells had a markedly positive ascorbate cyanide test. The mother of case 1 had an ascorbate cyanide test intermediate between her daughter and the normal control, while the result in the mother of case 2 was normal.

The patients' red cell pentose phosphate shunt activity was compared to that of normal and high reticulocyte controls (Table 1). Before new methylene blue stimulation, the pentose shunt activity of the patients' red cells was higher than that of the normal controls (\(p < 0.05\)) but was similar to that of the high reticulocyte controls. However, after stimulation, the patients' RBC pentose shunt activity was the same as that of the normal controls and was significantly decreased (\(p < 0.05\))

<table>
<thead>
<tr>
<th>Table 1. Red Cell Pentose Phosphate Shunt Activity in Pyrimidine 5'-Nucleotidase (5'-N) Deficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pentose Shunt Activity ((\mu)mol/Grx Oxidized (10^6) RBC (1 \text{ hr}^{-1}))</td>
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<tr>
<td>---------------------------------</td>
</tr>
<tr>
<td>Intact red cells</td>
</tr>
<tr>
<td>After stimulation*</td>
</tr>
<tr>
<td>Hemolysate</td>
</tr>
</tbody>
</table>

*Stimulation by \(10^{-4}\) M new methylene blue.
NS, not significant.
compared to the high reticulocyte controls. The patients' mothers had normal pentose shunt activity (data not shown).

Since the patients had lower than expected pentose phosphate shunt activity after new methylene blue stimulation and because the pentose phosphate shunt functions under severe restraint in the intact red cell, pentose phosphate shunt activity was measured in red cell hemolysates to determine if the decrease in shunt activity in the intact erythrocyte was due to increased shunt suppression or a loss of metabolic capacity. In the red cell hemolysates, the patients' pentose phosphate shunt activity was threefold that of the normal controls (p < 0.001) and was similar to that of the high reticulocyte controls (Table 1), suggesting increased shunt suppression in the intact RBC with pyrimidine 5'-nucleotidase deficiency.

**Intracellular pH**

The intraerythrocytic pH (pHi), extracellular pH, and transmembrane pH for the patients, their mothers, and the normal controls are shown in Table 2. Although all 3 groups had similar plasma pHs, the patients with pyrimidine 5'-nucleotidase deficiency had a decreased intraerythrocytic and an increased transmembrane pH.

**Effects of Pyrimidine 5'-Nucleotides on the Activity of Red Cell Enzymes**

Since the erythrocyte in pyrimidine 5'-nucleotidase deficiency appears to have a reversible suppression of pentose phosphate shunt activity, we evaluated the effects of increased pyrimidine 5'-nucleotide concentrations on red cell enzyme activity. The effects of cytidine 5'-nucleotides (CMP, CDP and CTP) on the activity of G6PD in red cell hemolysates at pH 7.1 (the pH of the red cells of case 1) is shown in Fig. 1 and Table 3. These cytidine nucleotides significantly inhibited the activity of G6PD. Most effective was 5.5 mM CTP, which decreased G6PD activity by 42.8%. The activity of G6PD was decreased by 28.2% and 14.6%, respectively, in the presence of 5.5 mM CDP or CMP. 6PGD activity was not affected by CTP, CDP, or CMP.

In order to confirm the inhibitory effects of CTP on G6PD, we studied the effects of this compound on the enzyme at various concentrations (Fig. 2). The activity of G6PD decreased with increasing concentrations of CTP.

Lineweaver-Burk plots of G6PD activity with G6P and NADP are shown in Fig. 3 (A and B). CTP was shown to inhibit the enzyme competitively with G6P, and noncompetitively with NADP. The K_i values were estimated to be 1.78 mM for G6P and 7.8 mM for NADP at pH 7.1.

**Effects of CTP on the Activity of G6PD and 6PGD at Various pHs**

The effects of CTP on G6PD and 6PGD activity were studied at pHs ranging from 6.6 to 7.8 and are shown in Fig. 4. G6PD activity declined in a linear fashion as the pH decreased (Fig. 4A). At any given pH, there was a further decline in G6PD activity in the presence of 5.5 mM CTP. Conversely, even though the activity of 6PGD declined with a decrease in pH, there was no further suppression of 6PGD activity in the presence of CTP (Fig. 4B).

**Effects of Various Pyrimidine 5'-Nucleotides on G6PD and 6PGD**

The effects of various pyrimidine 5'-nucleotides (5 mM) on G6PD and 6PGD activity at pH 7.1 are summarized in Table 3. Triphosphate nucleotides (CTP, UTP, and TTP) were the most effective inhibitors of G6PD activity. G6PD activity was inhibited 42.8%, 50%, and 52.5% in the presence of CTP, UTP, and TTP, respectively. For all 3 nucleotide bases, the inhibitory effect on G6PD activity was greater for the triphosphate than for the diphosphate or monophos-

### Table 2. Intraerythrocytic and Plasma pH in Pyrimidine 5'-Nucleotidase (PS'N) Deficiency

<table>
<thead>
<tr>
<th></th>
<th>PS'N Deficient</th>
<th>Mothers</th>
<th>Controls for</th>
<th>PS'N Deficient</th>
<th>Mothers</th>
<th>Controls for</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Case 1</td>
<td>Case 2</td>
<td>Case 1</td>
<td>Case 2</td>
<td>Case 1</td>
<td>Case 2</td>
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<tr>
<td>Extracellular pH</td>
<td>7.36</td>
<td>7.33</td>
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<td>7.33</td>
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<tr>
<td>Intraerythrocytic pH</td>
<td>7.10</td>
<td>7.18</td>
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<td>7.20</td>
<td>7.21</td>
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<tr>
<td>Transmembrane pH</td>
<td>0.28</td>
<td>0.17</td>
<td>0.15</td>
<td>0.11</td>
<td>0.17</td>
<td>0.13</td>
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</table>
Fig. 2. Effect of various final concentrations of CTP on G6PD activity. The experimental conditions are the same as in Fig. 1, except that different concentrations of CTP were added to the reaction mixture.

Fig. 3. Inhibitory effect of CTP on the activity of G6PD expressed as Lineweaver-Burk plots for substrates G6P and NADP. The initial velocity of G6PD was measured changing the concentrations of G6P or NADP under the same conditions as in Fig. 1. The reaction was started by the addition of 225 μM NADP or 225 μM G6P (final concentration). (A) G6P, (B) NADP (● 5.5 mM CTP, △ no CTP).

Table 3. Effects of Various Pyrimidine 5'-Nucleotides on the Activity of G6PD and 6PGD*

<table>
<thead>
<tr>
<th></th>
<th>G6PD μ mole · min⁻¹ · gHb⁻¹</th>
<th>Percent Inhibition</th>
<th>6PGD μ mole · min⁻¹ · gHb⁻¹</th>
<th>Percent Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>9.60</td>
<td>—</td>
<td>2.72</td>
<td>—</td>
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<tr>
<td>CTP</td>
<td>5.49</td>
<td>42.8</td>
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<tr>
<td>CDP</td>
<td>6.89</td>
<td>28.2</td>
<td>2.72</td>
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</tr>
<tr>
<td>CMP</td>
<td>8.20</td>
<td>14.6</td>
<td>2.72</td>
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<tr>
<td>UTP</td>
<td>4.80</td>
<td>50.0</td>
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<tr>
<td>UDP</td>
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<tr>
<td>UMP</td>
<td>8.21</td>
<td>14.5</td>
<td>2.72</td>
<td>0</td>
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<tr>
<td>TTP</td>
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<td>52.5</td>
<td>2.32</td>
<td>14.7</td>
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<tr>
<td>TDP</td>
<td>5.28</td>
<td>45.0</td>
<td>2.80</td>
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<tr>
<td>TMP</td>
<td>7.19</td>
<td>25.1</td>
<td>2.71</td>
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<tr>
<td>CTP + ATP</td>
<td>2.18</td>
<td>77.3</td>
<td>—</td>
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</table>

*The composition of reaction mixture and experimental conditions are the same as in Fig. 1.
DISCUSSION

A chronic nonspherocytic hemolytic anemia occurs in pyrimidine 5'-nucleotidase deficiency, the nature of which has been obscure. Associated clinical findings include splenomegaly and exacerbation of the anemia with infection, stress, or pregnancy.2 Previous investigations have shown: (1) increased Heinz body formation after the incubation of red cells with acetylphenylhydrazine, but not in fresh preparations,3 (2) unstimulated pentose phosphate shunt activity similar to that of normal reticulocyte controls,3,4 and (3) normal activity of the enzymes of the Embden-Meyerhof pathway, G6PD, 6PGD, glutathione reductase, and glutathione peroxidase in red cell hemolysates.5

The current investigations of erythrocytes with pyrimidine 5'-nucleotidase deficiency have confirmed Valentine's3 observation of an increase in Heinz body formation upon the exposure of affected cells to oxidant stress. In order to explain this phenomenon, pentose phosphate shunt activity was examined in intact red cells before and after new methylene blue stimulation and also in red cell hemolysates. The data from patients with pyrimidine 5'-nucleotidase deficiency were compared to those from control populations with both normal and elevated reticulocyte counts, since pentose phosphate shunt activity has been shown to be a cell age-related phenomenon.6 Although pentose phosphate shunt activity in pyrimidine 5'-nucleotidase deficiency was shown to be similar to that of normal reticulocyte controls after new methylene blue stimulation, it was significantly decreased compared to that of high reticulocyte controls (p < 0.02). However, there was no significant difference between the pentose shunt activities of the red cell hemolysates from the patients and from the high reticulocyte controls. These data suggest that there is increased suppression of pentose phosphate shunt activity in the intact red cell in pyrimidine 5'-nucleotidase deficiency, but that the suppressing factor(s) is lost or diluted out in tests performed in red cell hemolysates. This observation may explain the normal hemolysate G6PD activity noted in previous reports.5,6

The pentose shunt activity of the erythrocytes of case 2 was twice that of case 1 and approached the range of our high reticulocyte controls. This finding may be related to the patient's young age. All of our high reticulocyte controls were adults with hemolytic anemia. Travis has shown increased G6PD, hexokinase, and pyruvate kinase activity in the red cells of 11–12 mo old infants compared to adults. Thus, the red cells of a 2.5-yr-old child might be expected to have increased pentose shunt activity when compared to those from an adult population. The role of the spleen must also be considered when comparing these 2 patients. Since case 1 had previously undergone a splenectomy, a larger number of her most severely affected cells might remain in her peripheral circulation, while cells with a similar degree of dysfunction would be sequestered and destroyed in the spleen of case 2.

Since red cell concentrations of the pyrimidine 5'-nucleotides CTP, CDP, UTP, and UDP have been shown to be increased in hereditary pyrimidine 5'-nucleotidase deficiency,18 these nucleotides were con-
considered to be prime candidates for inhibitors of the pentose phosphate shunt. Since phosphorylated pyrimidine nucleotides do not cross the red cell membrane, we have shown that 5.5 mM CTP and 5.5 mM UTP have a marked inhibitory effect on the generation of $^{14}$CO$_2$ by the pentose phosphate shunt in red cell hemolysates.

The data presented indicate that the suppression of pentose phosphate shunt activity in the patients' red cells can be attributed to the inhibition of G6PD, the rate-limiting enzyme of the pentose phosphate shunt, by pyrimidine 5'-nucleotides. As shown in Figs. 1-4 and Table 3, CTP, CDP, UTP, UDP, TTP, and TDP significantly inhibit G6PD activity. The mode of inhibition is competitive with G6P, and noncompetitive with NADP (Fig. 3A and 3B). This result is consistent with the data of Yoshida showing that ATP, another nucleotide, is a competitive inhibitor of glucose-6-phosphate. These nucleotides, however, did not affect the activity of 6PGD (Table 3 and Fig. 4B), glutathione reductase, or glutathione peroxidase. Since Oda and Tanaka have shown that these pyrimidine 5'-nucleotides do not inhibit the activities of the glycolytic enzymes hexokinase, phosphofructokinase, and pyruvate kinase, their inhibitory effect appears to be specific for G6PD.

Based on reports of Torrance and Whittaker and Valentine et al., the estimated pyrimidine nucleotide concentration in affected red cells is in the range of 3.4-6.4 mM. The estimated red cell concentration of NADP is about 1 μM and the $K_m$ of G6PD for NADP is about 5 μM. Similarly, the G6P concentration is about 27 μM and the $K_m$ about 50 μM. Therefore, in vivo red cell G6PD should not be saturated by these substrates. Furthermore, since the $K_s$ of the CTP for G6P and NADP are approximately 1.7 mM and 7.8 mM, respectively (Fig. 3, A and B), the intraerythrocytic concentration of total pyrimidine 5'-nucleotides should be above the $K_s$ for G6P and approaching that of NADP. Thus, these data strongly suggest that inhibition of G6PD activity in pyrimidine 5'-nucleotidase deficient red cells should occur in vivo.

Yoshida indicated that G6PD activity will be inhibited 40% by physiologic concentrations of ATP (1.5 mM) in normal human red cells. Therefore, in red cells with pyrimidine 5'-nucleotidase deficiency, where the ATP concentration is normal, G6PD will be further suppressed by the combined effects of ATP and pyrimidine 5'-nucleotides. In our experimental system, G6PD activity was markedly suppressed in the presence of both ATP and CTP (Table 3).

Duhm showed that the intracellular pH (pHi) of red cells is decreased when impermeable anions, such as organic phosphates, accumulate in the cells. This fact suggested that the pHi of the patients' red cells should be decreased, which was confirmed in the present study. The decrease of pHi may induce some metabolic changes in the patients' red cells including suppression of the pentose shunt. As shown in Fig. 4, G6PD and 6PGD activities are inhibited by the decrease of pHi. This effect is accentuated for G6PD in the presence of 5.5 mM CTP.

The nature of the paradoxical increase in the reduced glutathione content of the pyrimidine 5'-nucleotidase deficient red cell remains enigmatic, since one would expect a decrease in reduced glutathione content, as is seen in hereditary G6PD deficiency with chronic hemolysis. Kondo et al. have demonstrated that the pyrimidine 5'-nucleotides inhibit the ATP-dependent transport of oxidized glutathione across the red cell membrane, which would serve to increase the total intraerythrocytic glutathione content. In addition, the decreased intraerythrocytic pH and concomitant increase in hydrogen ion concentration could shift the equilibrium between reduced and oxidized glutathione, favoring a further increase in reduced glutathione content. Finally, it should be remembered that an increased reduced glutathione content does not preclude the presence of impaired pentose phosphate shunt function. The potential reduced glutathione content of an erythrocyte with impaired pentose phosphate shunt and decreased intracellular pH might be even higher than that attained in pyrimidine 5'-nucleotidase deficiency, if there was normal function of the pentose phosphate shunt.

In conclusion, the hemolysis in pyrimidine 5'-nucleotidase deficiency appears to be due, in part, to a decrease in G6PD activity with subsequent suppression of pentose phosphate shunt activity. The mechanisms involved include: (1) competitive inhibition of G6P and noncompetitive inhibition of NADP for G6PD by the pyrimidine 5'-nucleotides and (2) further suppression of G6PD and 6PGD activity by a decrease in intraerythrocytic pH due to the accumulation of acidic pyrimidine 5'-nucleotides. Thus, the decreased pentose phosphate shunt activity should render the pyrimidine 5'-nucleotidase deficient red cell more susceptible to oxidant stress, Heinz body formation, and hemolysis.

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