Hereditary Nonspherocytic Hemolytic Anemia due to a New Hexokinase Variant With Reduced Stability

By Mauro Magnani, Vilberto Stocchi, Luigi Cucchiarini, Giuseppe Novelli, Sergio Lodi, Luciano Isa, and Giorgio Fornaini

A 27-year-old woman with severe chronic hemolytic anemia was found to have reduced red cell hexokinase activity when the degree of reticulocytosis was considered. This enzyme had normal pH-dependent activity, normal Km for glucose, fructose, and mannose, normal Km for Mg adenosine triphosphate (ATP)2 and K, for glucose-1,6-diphosphate. Furthermore, the pH-dependence and orthophosphate dependence of K, for glucose-1,6-diphosphate were normal. However, this hexokinase was inactivated rapidly at 44°C. No abnormalities were found in the red cell hexokinase isozymic pattern when it was compared with the profile obtained from cells of similar age. The hexokinase specific activity was reduced in all the red blood cell fractions obtained by density gradient ultracentrifugation; a marked difference in the distribution of cells through the gradient was evident. Among the glycolytic intermediates, a significant decrease of 2,3-diphosphoglycerate was evident. ATP and glucose 6-phosphate were also reduced when compared with cells of similar age. Glucose consumption of the hexokinase-deficient cells decreased, but the rate of glucose metabolized through the hexose monophosphate shunt was unchanged. Although the total hexokinase activity in lymphocytes was only reduced by 37%, a marked hexokinase deficiency was detected in blood platelets (20% to 25% of normal activity). The parents and one of two siblings of the patient were heterozygous for the defect, with 66% to 74% of normal erythrocyte hexokinase activity and reduced heat stability of the enzyme. These results, when compared with those obtained in previously reported cases of hexokinase deficiency, provide further evidence of the broad phenotypic variability that characterizes this disorder. Furthermore, it is suggested that failure of energy generation is probably the primary cause of hemolytic anemia in hexokinase deficiency.

CASE REPORT

The patient, a 27-year-old woman, was the first of three daughters of nonconsanguineous parents. Past history revealed uncomplicated term pregnancy and delivery. She was noted to be anemic from birth with mild hepatomegaly and marked splenomegaly. In her early infancy, several hospitalizations were necessary to perform blood transfusions. When she was admitted at 6 months of age, her liver was palpable 4.5 cm and her spleen was palpable 5 cm below the costal margin. The Hb was 5.3 g/dL, erythrocytes 2.1 x 1012/L, leukocytes 24 x 109/L, reticulocytes 1%, total bilirubin 0.5 mg/dL, and red cell osmotic fragility was slightly increased. Peripheral blood smears revealed anisopoikilocytosis with microcytosis, target cells, Howell-Jolly bodies, and five to ten normoblasts per 100 white cells. Bone marrow examination showed erythropoietic hyperplasia. Diagnosis of congenital hemolytic jaundice was made, and when she reached the age of 11 months, splenectomy was performed. Her subsequent course was characterized by normal growth and development, but repeated blood transfusions were required until she was 10 years old to maintain satisfactory hemoglobin levels. Scleral icterus was always present, with exacerbations during infections or occasional drug intake. The most recent (at the time of writing) hematological data were obtained in February 1984 when the patient, gravida one at the 15th week of pregnancy, was admitted because of fatigue and malaise.

Physical examination revealed hepatomegaly (3 cm below costal margin). Laboratory results were: Hb 8.4 g/dL, erythrocytes 2.4 x 1012/L, hematocrit 27%, mean corpusco
lar volume 132 fl, leukocytes 14.9 × 10⁶/L, platelets 780 × 10⁹/L, and reticulocytes 45%. The blood smear showed anisopoikilocytosis with crenated microspherocytes, basophilic stippling, and Howell-Jolly bodies. Total serum bilirubin was 3.7 mg/dL with 1.0 direct. Osmotic fragility tests and autohemolysis were normal. Coombs, Ham, and sucrose lysis tests were negative. No Heinz bodies were demonstrated. Estimation of Hb-A₂ was normal; Hb-F was slightly increased, and there were no abnormal bands on electrophoresis; no heat-labile hemoglobins were present. The family showed: father-Hb 14.7 g/dL, reticulocytes 1.3%; mother-Hb 13.2 g/dL, reticulocytes 0.6%; sister I-Hb 12.8 g/dL, reticulocytes 0.7%; and sister II—Hb 13.3 g/dL, reticulocytes 0.8%. None had morphological abnormalities. The patient and family members were evaluated by examination of glycolytic enzymes and erythrocyte metabolism (see Results).

MATERIALS AND METHODS

Venous blood from the proposita, relatives, and controls was collected in heparin (for lymphocyte and erythrocyte isolation) and acid-citrate-dextrose (for platelet isolation). Three milliliters of heparinized blood was precipitated immediately after withdrawal by adding an equal volume of 7.5% (vol/vol) HClO₄ and processed as suggested for the determination of glycolytic intermediates. One milliliter of heparinized blood was mixed with an equal volume of cold 0.5 mol/L of KOH and centrifuged at 2,500 rpm for 15 minutes at 4 °C on CF-50A Centriflo membrane cones (Amicon Corp, Mass) for the determination of reduced and oxidized nucleotides.

Red cells were isolated by filtering whole blood through a-cellulose-microcrystalline cellulose columns. Lymphocytes were prepared after density centrifugation of blood on Lymphoprep (Nycodenz, Oslo) for 30 minutes at 1,600 rpm, and at 4 °C. Monocytes were removed by adherence to plastic Petri dishes. Platelets were prepared after density centrifugation of blood on lymphoprep and the amount metabolized through the pentose shunt were determined as previously described.

Hexokinase isozyme pattern. One milliliter of hemolysate prepared as previously described was chromatographed on DE-52 columns (Whatman, Maidstone, England) (0.3 × 24 cm) equilibrated in 5 mmol/L sodium potassium phosphate buffer, pH 7.5, containing 0.25 mmol/L glucose and 1 mmol/L dithiothreitol. The columns were prepared with 280 mL of a linear gradient of KCl from 0 to 0.4 mol/L in the same buffer and operated at 5 mL/h. Fractions of 0.7 mL were collected and assayed for HK activity. Further details of the method can be found in a previous paper.

Hexokinase purification. For kinetic experiments, the red cell hexokinase was partially purified by DE-52 column chromatography. Three milliliters of hemolysate was applied to a 1.2 × 4.0-cm DE-52 column equilibrated in 5 mmol/L sodium potassium phosphate buffer, pH 7.5, containing 3 mmol/L of KF, 3 mmol/L mercaptoethanol, 0.25 mmol/L glucose (buffer A). The column was washed with 3 vol of buffer A, and the enzyme was eluted with 0.4 mol/L KCl in the same buffer. This procedure removes the bulk of platelet-rich plasma on Sepharose 2B columns (Pharmacia, Uppsala, Sweden).

Determination. Enzymes and intermediate compounds were determined by the Beutler methods at 37 °C and 2,3-diphosphoglycerate, using the Boehringer (Mannheim, FRG) test kit. Glutathione peroxidase was determined as described, glutathione-S-transferase, glyoxalase I and II, and purine nucleoside phosphorylase, and uridine diphosphate glucose phosphophorylase were determined by the methods in the cited references. Platelets and white cell lysates were obtained by sonication at 100 W for 20 seconds at 0 °C in the presence of 5 mmol/L of sodium potassium phosphate buffer, pH 7.4, containing 3 mmol/L of mercaptoethanol, 3 mmol/L of KF, 1 mmol/L of glucose, 1 mmol/L of dithiothreitol, 0.4% (wt/vol) saponin, 0.5% (vol/vol) Triton X-100, and 1 mmol/L of phenylmethylsulfonylfluoride. The sonicate was centrifuged for 15 minutes in an Eppendorf Microfuge, and the high-speed supernatant was utilized for enzyme and protein assay.

Reduced and oxidized nucleotides, ATP, ADP, and AMP, were determined by a high-performance liquid chromatographic method. Details of the extraction procedures, recoveries, and reproducibility of the methods used are described elsewhere.

Glucose metabolism. Glucose utilization by intact red blood cells and the amount metabolized through the pentose shunt were determined as previously described.

Table 1. RBC Enzyme Activities in the Proposita, Relatives, and Control Subjects

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Proposita</th>
<th>Father</th>
<th>Mother</th>
<th>Sister I</th>
<th>Sister II</th>
<th>Normal Control Subjects</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hexokinase</td>
<td>0.87 ± 0.30*</td>
<td>0.73</td>
<td>0.67</td>
<td>1.01</td>
<td>0.65</td>
<td>0.98 ± 0.16</td>
</tr>
<tr>
<td>Glucose phosphate isomerase</td>
<td>84.83</td>
<td>72.98</td>
<td>58.31</td>
<td>52.41</td>
<td>74.59</td>
<td>60.8 ± 11.00</td>
</tr>
<tr>
<td>Phosphofructokinase</td>
<td>11.82</td>
<td>11.85</td>
<td>10.46</td>
<td>9.53</td>
<td>11.25</td>
<td>11.0 ± 2.30</td>
</tr>
<tr>
<td>Aldolase</td>
<td>5.28</td>
<td>2.72</td>
<td>2.62</td>
<td>2.66</td>
<td>3.31</td>
<td>3.19 ± 0.86</td>
</tr>
<tr>
<td>Triose phosphate isomerase</td>
<td>2.665</td>
<td>1.729</td>
<td>1.600</td>
<td>2.460</td>
<td>1.905</td>
<td>2.200 ± 350</td>
</tr>
<tr>
<td>Glyceraldehyde phosphate dehydrogenase</td>
<td>175</td>
<td>156</td>
<td>144</td>
<td>151</td>
<td>226</td>
<td>200 ± 40</td>
</tr>
<tr>
<td>Phosphoglycerate kinase</td>
<td>386</td>
<td>315</td>
<td>308</td>
<td>332</td>
<td>318</td>
<td>320 ± 36</td>
</tr>
<tr>
<td>Monophosphoglyceromutase</td>
<td>20.10</td>
<td>18.73</td>
<td>18.09</td>
<td>17.88</td>
<td>17.88</td>
<td>18.0 ± 3.80</td>
</tr>
<tr>
<td>Diphosphoglyceromutase</td>
<td>4.15</td>
<td>5.25</td>
<td>4.75</td>
<td>4.75</td>
<td>5.05</td>
<td>4.78 ± 0.60</td>
</tr>
<tr>
<td>Enolase</td>
<td>5.91</td>
<td>4.66</td>
<td>4.19</td>
<td>4.41</td>
<td>5.01</td>
<td>5.20 ± 0.80</td>
</tr>
<tr>
<td>Pyruvate kinase</td>
<td>29.08</td>
<td>18.62</td>
<td>15.74</td>
<td>16.05</td>
<td>22.08</td>
<td>14.0 ± 2.00</td>
</tr>
<tr>
<td>Lactate dehydrogenase</td>
<td>208</td>
<td>180</td>
<td>175</td>
<td>189</td>
<td>174</td>
<td>180 ± 25</td>
</tr>
<tr>
<td>Glucose-6-phosphate dehydrogenase</td>
<td>14.20</td>
<td>9.61</td>
<td>8.38</td>
<td>8.72</td>
<td>10.05</td>
<td>8.5 ± 2.50</td>
</tr>
<tr>
<td>6-Phosphogluconate dehydrogenase</td>
<td>11.28</td>
<td>8.12</td>
<td>7.12</td>
<td>6.76</td>
<td>10.52</td>
<td>8.2 ± 0.50</td>
</tr>
</tbody>
</table>

*Values are expressed as U/g hemoglobin and represent means ± SD (N = 5).

*Reticulocyte percentages on blood collected on four different occasions were found to range from 26% to 45%.
Table 2. Some Kinetic Properties of Partially Purified Hexokinase
From the Proposita and Controls

<table>
<thead>
<tr>
<th>Hexokinase</th>
<th>Proposita</th>
<th>Normal Control Subjects</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_m$ glucose ($\mu$mol/L)</td>
<td>$44 \pm 3.00$</td>
<td>$47 \pm 3.00$</td>
</tr>
<tr>
<td>$K_m$ fructose (mmol/L)</td>
<td>$10 \pm 1.50$</td>
<td>$12 \pm 1.50$</td>
</tr>
<tr>
<td>$K_m$ mannose (mmol)</td>
<td>$0.08 \pm 0.01$</td>
<td>$0.07 \pm 0.01$</td>
</tr>
<tr>
<td>$K_m$ Mg ATP (mmol/L)</td>
<td>$0.60 \pm 0.04$</td>
<td>$0.59 \pm 0.04$</td>
</tr>
<tr>
<td>$K_m$ glucose-1,6-diphosphate ((\mu)mol/L)</td>
<td>$22 \pm 2$</td>
<td>$22 \pm 2.00$</td>
</tr>
</tbody>
</table>

The $K_m$ for Mg ATP was determined at 5 mmol/L glucose, and the $K_m$ for hexoses was determined at 5 mmol/L Mg ATP. All experiments were performed in 80 mmol/L of Tris-HCl, pH 7.20, at 37 °C.

hemoglobin and provides a 40-fold to 50-fold purification with complete recovery of the enzyme.

RBC fractionation. RBCs were separated into fractions of different mean age by ultracentrifugation through a density gradient of Ficoll-Triosil layers (Pharmacia, Uppsala, Sweden; Nyegaard, Oslo) as previously reported. After centrifugation, the red cells were separated into six fractions and numbered from 1 to 6, representing erythrocytes of increasing density (age).

RESULTS

RBC enzyme levels. Activities of the same red cell enzymes in the proposita, relatives, and controls are reported in Table 1. The HK specific activity of the proposita was near the lower limit of normal controls, while all other cell age-dependent enzymes were increased (pyruvate kinase, glucose 6-phosphate dehydrogenase, aldolase, adenylate kinase, and glutamate-oxalacetate transaminase). Both parents and one sibling were found to have HK activity ranging from 66% to 74% of normal. The activities of glutathione reductase, glutathione peroxidase, glutathione-S-transferase, glosomalase I and II, adenosine deaminase, purine nucleoside phosphorylase, and uridine-diphosphoglucosephosphorylase were all in the normal range. The HK activity of reticulocyte-rich (30% to 40%) blood samples obtained by density-gradient ultracentrifugation from the blood of normal subjects was found to be $4.26 \pm 0.65$ U/g hemoglobin (mean $\pm$ SD, $n = 4$). These results provide clear evidence of a hexokinase deficiency in the patient. The calculated HK, glucose 6-phosphate dehydrogenase activity ratio was 0.0612 in the proposita and 0.236 in reticulocyte-rich blood samples, a further confirmation of this deficiency.

Enzyme properties. The kinetic properties of partially purified HK from the proposita and controls are reported in Table 2. No kinetic abnormalities could be detected in the

![Fig 1](https://example.com/fig1.png)

Fig 1. (A) Influences of orthophosphate on the inhibition of hexokinase by 100 $\mu$mol/L of glucose-1,6-diphosphate. The experiments were performed in 80 mmol/L of Tris-HCl pH 7.2, and at 37 °C. The concentration of glucose was 5 mmol/L, free Mg$^{2+}$ 5 mmol/L, and Mg ATP 1 mmol/L. Orthophosphate (Pi) was added as sodium potassium phosphate at pH 7.2. $V_{max}$ is the reaction velocity in the presence of the inhibitor and orthophosphate, $V_{max}$ equals the reaction velocity in the presence of inhibitor and absence of Pi, and $V_{max}$ equals the reaction velocity in the absence of inhibitor and Pi. (B) Influence of pH in the inhibition of hexokinase by 50 $\mu$mol/L glucose-1,6-diphosphate. Other conditions as in (A) except that the pH was as indicated. Proposita (○); normal control subjects (△).

![Fig 2](https://example.com/fig2.png)

Fig 2. Heat stability of partially purified hexokinase from the proposita (open symbols) and normal control subjects (filled symbols). Experiments were performed at 44 °C in 5 mmol/L of sodium potassium phosphate buffer, pH 7.5, 3 mmol/L of mercaptoethanol, 1 mmol/L of dithiotreitol, and glucose 5 mmol/L (●, ○). Hexokinase activity was 0.2 U/mL.
partially purified enzyme from the proposita and heterozygous subjects. Because inorganic phosphate (Pi) is able to overcome partially the inhibition of glucose-1,6-diphosphate on HK, we investigated this effect on the enzyme of our patient (Fig 1). Contrary to what was found in a previous case of HK deficiency,16 there were no abnormalities. A normal pH-dependence of glucose-1,6-diphosphate inhibition of this hexokinase was observed.

The stability of partially purified HK at 44 °C indicated that the enzyme from the proposita was not as stable as that of normal controls (Fig 2). This instability is also glucose concentration-dependent. Heterozygous subjects have a HK activity decay profile in between that for the proposita and controls (results not shown).

The pH dependence of HK activity in glycylglycine buffer (0.130 mol/L) did not differ from the pH profile of normal control subjects. A broad optimum was found from pH 7.75 to 8.50.

Hexokinase isozymic pattern. Human red cell hexokinase exists in multiple molecular forms that are subtypes of HK 1.23,8 These isozymic forms are separable by ion exchange chromatography and have been designated HK Ia, Ib, and Ic. The patient’s erythrocytes contained an increased amount of HK Ib (Fig 3) as compared with that of normal control subjects. Among these isozymes, however, HK Ib is the most cell age-dependent, so that the isozymic pattern of the proposita appears normal when compared with that of young cells previously reported.22

Hexokinase activity and RBC age. It is well known that HK activity depends strictly on RBC age,30,21,39-41 and this fact must be considered when one is studying the enzyme in patients with hemolytic anemia. Because it is important to have information not only on the relative enzyme levels of cells of similar age, but also on the decay of enzymatic activity during cell aging, we fractionated the red cells of the patient, heterozygous subjects, and normal controls by ultracentrifugation on density gradients. Six cell fractions of increasing mean age were obtained. The HK and glucose-6-phosphate dehydrogenase activities of these cells are shown in Fig 4. These results provide evidence of reduced HK activity in all RBC fractions, indicating that the proposita and normal control HK show similar rates of decay during cell aging. The distribution of cells in the gradients is reported in Fig 5. As expected from the hematologic data, most of the cells of the proposita sediment were found in fraction 1, but a significant number of cells were also found in fraction 5. One possible reason for this cell distribution is the presence of two cell populations, one consisting mainly of reticulocytes (fraction 1) and the other of mature erythrocytes (fraction 5). This would be in agreement with the evidence obtained in pyruvate kinase deficiency,42 for which it has been shown that reticulocytes are sequestered and
destroyed more rapidly than mature erythrocytes. In other words, it has been proposed that many reticulocytes cannot meet the high ATP requirements of these cells, but others, probably those with the highest residual activity, reach maturity and survive normally despite their enzyme deficiency. This phenomenon seems to be common in many erythroenzymopathies of glycolysis.

Glycolytic intermediates and glucose metabolism. We have determined the red cell glycolytic intermediates in the proposita, relatives, and normal control subjects. Apparent-

Fig 4. Hexokinase and glucose 6-phosphate dehydrogenase (G-6-PDH) activities in red blood cells of different ages. Red blood cells from normal control subjects and the proposita were separated into six fractions of increasing mean age by ultracentrifugation through gradients of Ficoll-Triosil. After centrifugation, red cell fractions were collected into separate tubes, washed, hemolyzed, and assayed for hexokinase and G-6-PDH activities and hemoglobin concentrations. Fractions from 1 through 6 represent red cells of increasing density (age). Normal control subjects (A); proposita (D).

Fig 5. Separation of the proposita and normal control subject red blood cells in fractions of different mean ages by density-gradient ultracentrifugation. After centrifugation, red cell fractions were collected into separate tubes, diluted by isotonic NaCl, and counted. Results are expressed as the percentage of cells on each fraction with respect to the number of cells loaded onto the gradient. (A) proposita; (B) normal control subjects.

ly, only 2,3-diphosphoglycerate is significantly reduced in the erythrocytes of our patient, while other compounds (i.e., 3-phosphoglycerate, ATP, ADP, AMP) are increased (Table 3). However, if a comparison is made with the concentration of glycolytic intermediates in cells of similar age, the glucose 6-phosphate and ATP concentrations are also reduced. In fact, we found that the concentration of glucose 6-phosphate in cells of fraction 1 from normal controls, obtained by density-gradient ultracentrifugation (about 35% reticulocytes) was 55 ± 5 nmol/L, 2,3-diphosphoglycerate was 6.5 ± 0.5 mmol/L and ATP was 2.7 ± 0.3 mmol/L.

The glucose utilization by our patient's cells was strongly reduced, as in the heterozygous subjects. This is much more marked if considered in relation to the cell age. The amount of glucose utilized in the hexose monophosphate shunt does not show great differences (Table 4).

Oxidized and reduced nucleotides and glutathione. The levels of erythrocytes oxidized and reduced NADP⁺ and NAD⁺ and the erythrocyte content of reduced glutathione are reported in Table 5. To our knowledge, this is the first time that these values are provided in a case of enzymopathy of anaerobic glycolysis; the values suggest that in the case under consideration, no disturbance in the redox state of the cell occurs.

Hexokinase activity in lymphocytes and platelets. Activities of hexokinase and other glycolytic enzymes (not shown) were also determined in lymphocytes and platelets from the proposita. These results confirm that the HK deficiency is also present in platelets. The values found were
Hexokinase in the erythrocyte is of central importance because it is one of the rate-limiting steps in anaerobic glycolysis, the only pathway by which ATP is produced in these cells. Only 12 cases of HK deficiency in ten unrelated families have been described. This apparent rarity may be due, at least in part, to the marked age-dependence of the enzyme. In fact, the young red cell population of homozygous deficient subjects apparently masks the true severity of the enzyme deficiency, as documented in all cases described thus far. Taking this fact into account, the residual HK activity in the erythrocytes of the patient described is only 20% to 25% of normal, a value very close to that found in blood platelets. In other words, it seems that the evaluation of HK activity in platelets can be proposed as a method to establish whether or not HK-deficiency in erythrocytes exists. This method does not need any correction because of cell age and is quite simple to perform. Reduced HK activity in platelets of patients with red cell HK deficiency has been reported by others.

The residual enzyme in the erythrocytes of our patient shows normal kinetic and regulatory properties, but a reduced heat stability. The values found, however, differ from those reported by others. For example, the Km value for glucose of the enzyme from normal subjects reported by Paglia et al is 0.25 mM, and the value reported by Newman et al is 0.212 mM. On the other hand, it is commonly accepted that the Km for glucose of human erythrocyte HK is in the 0.047 to 0.062 mM range. These discrepancies can probably be explained by the use of inappropriate samples, ie, red cell lysate instead of partially purified enzyme, or inappropriate substrate concentrations (up to 10 mM). Similarly, as we show in Fig 1 and in agreement with others, the inhibition of HK by glucose-1,6-diphosphate and its reversal by orthophosphate, are strongly pH-dependent. This fact must be considered carefully when the effect of glucose-1,6-diphosphate or orthophosphate is examined to avoid further discrepancies in the literature about the properties of HK. Among the cases of HK deficiency described in the literature, only those reported by Keitt, Board et al, and Newman et al show reduced heat stability of the enzyme. However, the enzyme of the patient described by Keitt also possesses an increased Km for glucose, and that described by Newman et al shows a decreased Km for glucose. Apparently our case is similar to that described by Board et al, but these authors have not provided other information about the kinetic properties of the enzyme except for the Km for glucose and ATP. Furthermore, the clinical manifestations of red cell HK deficiency are more severe in our patient, as compared with the case described by Board et al. In our patient, the minimum

25.8 and 22.0 mU/mg protein, respectively (normal values for lymphocytes, 40±9; platelets, 102±10 mU/mg protein). The most probable explanation of the slightly reduced lymphocyte HK activity is the compensation of the deficiency by the proportional increase of HK type III, as Rijksen et al have shown.

### Discussion

Hexokinase in the erythrocyte is of central importance because it is one of the rate-limiting steps in anaerobic glycolysis, the only pathway by which ATP is produced in these cells. Only 12 cases of HK deficiency in ten unrelated families have been described. This apparent rarity may be due, at least in part, to the marked age-dependence of the enzyme. In fact, the young red cell population of homozygous deficient subjects apparently masks the true severity of the enzyme deficiency, as documented in all cases described thus far. Taking this fact into account, the residual HK activity in the erythrocytes of the patient described is only 20% to 25% of normal, a value very close to that found in blood platelets. In other words, it seems that the evaluation of HK activity in platelets can be proposed as a method to establish whether or not HK-deficiency in erythrocytes exists. This method does not need any correction because of cell age and is quite simple to perform. Reduced HK activity in platelets of patients with red cell HK deficiency has been reported by others.

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### Table 3. RBC Glycolytic Intermediates

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Proposita</th>
<th>Father</th>
<th>Mother</th>
<th>Sister I</th>
<th>Sister II</th>
<th>Normal Control Subjects</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose-6-phosphate</td>
<td>32.3</td>
<td>19.4</td>
<td>16.7</td>
<td>20.7</td>
<td>17.7</td>
<td>28.0 ± 7.0</td>
</tr>
<tr>
<td>ATP</td>
<td>1.609</td>
<td>1.514</td>
<td>1.228</td>
<td>1.350</td>
<td>1.407</td>
<td>1.480 ± 0.80</td>
</tr>
<tr>
<td>ADP</td>
<td>178.0</td>
<td>88.0</td>
<td>84.0</td>
<td>79.0</td>
<td>88.0</td>
<td>85 ± 8.0</td>
</tr>
<tr>
<td>AMP</td>
<td>27.0</td>
<td>13.0</td>
<td>9.0</td>
<td>10.0</td>
<td>11.0</td>
<td>10 ± 1.5</td>
</tr>
<tr>
<td>2,3-Diphosphoglycerate</td>
<td>4,080</td>
<td>4,270</td>
<td>3,440</td>
<td>4,420</td>
<td>3,370</td>
<td>4,700 ± 200</td>
</tr>
</tbody>
</table>

All values are expressed as nmol/mL of RBCs. The concentrations of the other glycolytic intermediates not shown were in the normal range.

*Mean ± SD (n = 5).
percentage of reticulocytes found during six months of observation was 26% and the maximum was 45%, whereas Board's patient had reticulocyte counts ranging from 5% to 7.6%. In conclusion, it is probable that the HK deficiency we report is due to a new mutation that results in the production of an enzyme with reduced heat stability.

Our results provide further evidence of the broad genetic heterogeneity that characterizes the known erythroenzymopathies and is especially manifest in HK-deficiency. This phenotypic variability has been attributed to the multiple molecular forms of HK present in human erythrocytes on the assumption that these molecular forms of HK show different kinetic, regulatory, and physical properties. We have been able to show that at least the major forms of human erythrocyte HK possess similar kinetic and regulatory properties and similar thermostability. Therefore, different proportions of erythrocyte HK isoenzymes cannot confer different properties to HK and therefore cannot account for the phenotypic variability.

The metabolic consequences of the reduced HK activity on the erythrocyte are dramatic. The utilization of glucose, the concentrations of glucose 6-phosphate, ATP, and 2,3-diphosphoglycerate are all reduced when compared with the values for cells of similar age. The amount of glucose utilized in the hexose monophosphate shunt is normal or slightly increased, indicating that this metabolic pathway, under resting conditions, is not regulated by glucose 6-phosphate concentrations in the range reported. These data are also in agreement with the cellular concentrations of reduced glutathione and reduced nucleotides that are all in the normal range. In other words, the cellular redox state appears to be normal and not influenced by HK deficiency. It is important to comment briefly on the data reported in Table 5. These values differ from those of others, but the discrepancies arose from the methods used for the determination of reduced or oxidized nucleotides. We have used a new technique that provides recoveries of 90% of the nucleotides of interest and seems very accurate. Details of the procedure and a comparison with other methods are reported by Stocchi. Excluding the possibility of an oxidative hemolysis, the primary cause of hemolytic anemia in HK deficiency most probably lies in the failure of energy generation and metabolic depletion. An ATP concentration of about 50% of that found in cells of comparable age seems too low to meet the metabolic needs of HK-deficient cells adequately. The absolute ATP concentration in the proposita's erythrocytes is similar to that found in mature erythrocytes and is, therefore, apparently high enough to sustain the red cell requirements. However, two major processes not active in mature red cells account for the ATP consumption in reticulocytes. These are the synthesis of hemoglobin and ATP-dependent proteolysis. The energy cost of the first process has been estimated, while that of the ATP-dependent proteolysis is unknown at present.

Because the reticulocytes have a greater ATP consumption than do mature erythrocytes, and because glucose constitutes the main substrate of energy metabolism of reticulocytes, reduced HK activity is probably more critical at the reticulocyte stage than in mature erythrocytes. This mechanism could probably explain why erythrocytes survive better than reticulocytes in enzymopathies of anaerobic glycolysis. For purposes of identification, and in accordance with the recommendations by international standardization committees for G6PD and pyruvate kinase deficiencies, this defect is tentatively designated "HK-Melzo."

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