ATP Metabolism in Pyruvate Kinase Deficient Erythrocytes

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With the technical assistance of Wilma J. Hudson

The mature human erythrocyte, without a nucleus, mitochondria, or endoplasmic reticulum, has limited metabolic capabilities. It depends upon anaerobic glycolysis for biochemical energy. This leaves the erythrocyte little ability to compensate for metabolic defects. Pyruvate kinase (PK) deficiency is one of an increasing list of red cell enzyme deficiencies associated with congenital non-spherocytic hemolytic anemia.

Valentine, Tanaka and Miwa initially suggested ATP levels may be reduced in PK deficient erythrocytes since PK catalyzes one of two glycolytic reactions that participate in ATP generation. Their prediction has subsequently been confirmed and extended by reports of in vitro instability of ATP in PK deficient cells. Changes in electrolyte concentrations within the red cell and increased permeability of the cell membrane to potassium support the contention that hemolysis in this condition may result from insufficient energy to maintain a normal electrolyte gradient between the red cell and its environment.

A lack of ATP cannot completely explain the hemolysis associated with red cell PK deficiency because hemolysis also occurs when red cell ATP is normal, almost normal, or elevated. The mechanism whereby the addition of ATP to the incubation system corrects autohemolysis in this condition is difficult to relate with intracellular events since erythrocyte membranes are impermeable to ATP. Autohemolysis is not always corrected by the addition of ATP. Spherocytosis, which occurs when excessive demands are placed upon ATP reserves, as in hereditary spherocytosis and when ATP generation is artificially impaired in vitro, is not a feature of PK deficient erythrocytes. Conversely, acanthocytosis, which occurs in red cell PK deficiency, is not dependent upon...
abnormal ATP metabolism. These observations suggest that factors other than ATP deficiency contribute to the hemolysis of PK deficient erythrocytes.

The studies described in this paper, which have already been published in preliminary form, indicate that ATP deficiency is not essential for hemolysis of PK deficient red cells. Conversely, in vitro hemolysis in this condition seems related to a high energy phosphate-dependent metabolic process. Two of the five patients under study have been the subject of a previous clinical report.

METHODS

Blood was collected from 5 patients and 10 healthy adult controls under sterile conditions. Heparinized blood was used for pyruvate kinase assays and autohemolysis studies. Defibrinated blood was used for ATP assays, inorganic phosphate (\(P_i\)), Na and K determinations. Blood from members of Families A and C, accompanied by control samples, was shipped in insulated containers under wet ice to Dr. W. N. Valentine's laboratory for PK assay. Red cell \(P_i\), ATPase activity, Na and K were all measured on the same red cell preparations that had been washed three times in physiological saline. Hemolysis was achieved by freezing and thawing. Spectrophotometric measurements were performed on a Beckman DU spectrophotometer. Red cell survival was measured by the method of Cooper and Owen.

Autohemolysis. The method described by Young et al. was used with corrections made for variations in hematocrit. Incubations were for 48 hours in an air-dry oven at 37 C. Hematocrits were determined by the microhematocrit method. Hemoglobin levels were measured on whole blood by a cyanmethemoglobin method and on plasma by a benzidine method. In studying the effect of ATPase inhibitors upon autohemolysis, saline solutions of ouabain (Fougera Products, Hicksville, New York) and the sodium salts of ethacrynic acid (Merck, Sharp and Dohma Laboratories, West Point, Pennsylvania)* and of p-chloromercuribenzoic acid (Mann Research Laboratories, New York, New York) were used. One part saline solutions of these ATPase inhibitors in the molar concentrations listed were added to 20 parts of whole blood. These molar concentrations of ATPase inhibitors were selected from the extent of their hemolytic effect upon blood from a patient who has hereditary spherocytosis.

PK Assay. The method described by Tanaka, Valentine and Miwa was used. One unit represents the activity derived from 10\(^{10}\) erythrocytes to dehydrogenate 1 micromole NADH in one minute at 37 C.

ATP Assay. Hemolysates were deproteinized with perchloric acid immediately upon thawing to minimize exposure to plasma ATPase. An indirect UV assay was employed that measures ATP from the rate at which NAD is reduced (ATP assay kits, TC-J-15979, Boehringer Mannheim Corporation, New York, New York). Red cell ATP is expressed as micromoles/Gm. Hb.

\(P_i\) and ATPase Activity Determinations. Erythrocytes were washed 3 times in physiologic saline. Hemolysate Mg, Na and K dependent ATPase activity was assayed indirectly by measuring \(P_i\) release during one hour of incubation at 37 C. Hemolysates were agitated prior to adding to the assay systems so as to get an even distribution of red cell membranes. A modification of the method described by Harvald et al. was used. The incubation mixture contained 50 millimoles Tris buffer (pH 7.6), 3 millimoles ATP (Mann Research Laboratories, New York, New York; lot no. 2348), 6 millimoles MgCl\(_2\), 100 millimoles NaCl, 20 millimoles KCl and 1 ml. hemolysate in a fixed volume of 2 ml. Inorganic phosphate was measured by the method of Fiske and Subbarow.

Red Cell Na and K Measurements. One part hemolyzed red cells was diluted in 200 parts of a 15 mEq./L. lithium nitrate solution. Na and K were measured by flame photometry (Instrumentation Laboratories, Boston, Massachusetts, Model 143) and expressed as mEq. per liter of hemolyzed cells.

* Courtesy Daniel E. Duggan, Ph.D.
Table 1.—Routine Hematologic Findings in the 5 Patients under Study

<table>
<thead>
<tr>
<th>Family</th>
<th>Age/Sex</th>
<th>Hematocrit</th>
<th>Reticulocyte * Count (%)</th>
<th>RBC Survival</th>
<th>48 Hour Autohemolysis</th>
<th>Times Transfused</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>17/F</td>
<td>34</td>
<td>4</td>
<td>21.5</td>
<td>1.4</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>16/F</td>
<td>33</td>
<td>—</td>
<td>4.8</td>
<td>—</td>
<td>1.8</td>
</tr>
<tr>
<td>B</td>
<td>58/M</td>
<td>24</td>
<td>34</td>
<td>9.4</td>
<td>15</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>57/F</td>
<td>30</td>
<td>34</td>
<td>4</td>
<td>12.5</td>
<td>0</td>
</tr>
<tr>
<td>C</td>
<td>4/M</td>
<td>17</td>
<td>27</td>
<td>10.8</td>
<td>18</td>
<td>13</td>
</tr>
</tbody>
</table>

*Mean prespleenectomy.

RESULTS

Clinical and Genetic Findings

The severity of hemolysis varied considerably from patient to patient (Table 1). Erythrocyte morphology was normal in all 5 patients. When autohemolysis was increased, it was corrected with ATP but not with glucose. Red cell survival was shortened but splenic sequestration was not excessive in the 3 patients who were studied. Nevertheless, clinical and hematologic improvement followed splenectomy. Postsplenectomy reticulocyte counts have exceeded 50 per cent in the severely affected fifth patient. Reticulocytoses have not increased significantly in the other two patients who underwent splenectomy.

Biochemical genetic studies were performed on 22 members of the 3 families (Fig. 1). Red cell pyruvate kinase was markedly reduced in the 5 homozygotes, none being demonstrable in the most severely affected patient from Family C. Nine members had intermediate levels of enzyme, reflecting the heterozygous state. Heterozygotes had normal hematocrits and reticulocyte counts. These studies indicate that pyruvate kinase deficiency is inherited as an autosomal defect of partial penetrance. Its clinical expression is completely recessive.

Red Cell ATP Levels and Stability

Red cell ATP (Table 2) was significantly increased in all 5 patients (p <
Repeat determinations upon Family A homozygotes suggest these ATP levels fluctuate little. Control red cell ATP levels were relatively uniform (range: 1.51-2.04 micromoles/Gm. Hb). Similar values were obtained when the cells were washed prior to assaying ATP, indicating that the results obtained were not significantly influenced by brief exposure to plasma ATPases following thawing.

The absolute ATP decline in erythrocytes from 4 patients did not differ significantly from control values during 96 hours of incubation at 37C. (Table 2). After 72 hours of incubation, ATP also appeared stable in vitro in the fifth pa-
<table>
<thead>
<tr>
<th>Source</th>
<th>ATP (micromoles/liter)</th>
<th>Pi (micromoles/liter)</th>
<th>ATPase (1-hour incubation)</th>
<th>Na (meq/liter)</th>
<th>K (meq/liter)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Family A-I</td>
<td>2.46 ± 0.68</td>
<td>1.23 ± 0.14</td>
<td>26.7 ± 4.0</td>
<td>62.8 ± 7.1</td>
<td>82.4 ± 8.5</td>
</tr>
<tr>
<td>Family A-II</td>
<td>2.66 ± 0.14</td>
<td>1.32 ± 0.08</td>
<td>26.6 ± 4.0</td>
<td>67.2 ± 10.0</td>
<td>89.5 ± 11.2</td>
</tr>
<tr>
<td>Family B-I</td>
<td>2.96 ± 0.08</td>
<td>1.64 ± 0.09</td>
<td>21.2 ± 3.5</td>
<td>71.4 ± 12.3</td>
<td>86.0 ± 13.6</td>
</tr>
<tr>
<td>Family B-II</td>
<td>4.07 ± 0.03</td>
<td>1.84 ± 0.04</td>
<td>27.0 ± 4.0</td>
<td>73.2 ± 14.0</td>
<td>-</td>
</tr>
<tr>
<td>Family C-I</td>
<td>2.36 ± 0.12</td>
<td>1.48 ± 0.09</td>
<td>23.6 ± 3.8</td>
<td>67.3 ± 15.0</td>
<td>-</td>
</tr>
<tr>
<td>Patient Mean</td>
<td>2.90 ± 0.13</td>
<td>1.42 ± 0.11</td>
<td>26.3 ± 4.2</td>
<td>64.2 ± 13.3</td>
<td>-</td>
</tr>
<tr>
<td>± SD</td>
<td></td>
<td></td>
<td>± 0.18</td>
<td>± 0.03</td>
<td>± 0.07</td>
</tr>
<tr>
<td>10 Controls Mean</td>
<td>1.80 ± 0.14</td>
<td>1.35 ± 0.09</td>
<td>25.8 ± 4.1</td>
<td>66.2 ± 14.5</td>
<td>-</td>
</tr>
<tr>
<td>± SD</td>
<td></td>
<td></td>
<td>± 0.29</td>
<td>± 0.07</td>
<td>± 0.07</td>
</tr>
</tbody>
</table>

*p* Not statistically significant.
tient's erythrocytes. The per cent of fresh cell ATP levels remaining at the end of each of the 4 days of incubation and normalized least square regression lines derived from these values are shown on Figure 2. Blood obtained from Family A and Family B homozygotes yielded similar regression lines, which were only half as steep as the slope derived from 10 controls (M values). ATP depletion in PK deficient red cells was 36 per cent less than the mean control value following 96 hours of incubation.

A collection of blood from each Family A homozygote, with hematocrit adjusted to 50 per cent, was halved. A fixed volume of buffered cyanide was added to one half of this blood to a final concentration of 5 millimoles/ml. A similar volume of physiologic saline was added to the other half. After 48 hours of incubation, mean ATP levels from both patients were identical from both preparations. Incubation with cyanide was not associated with excessive autohemolysis. At the time blood was collected for this experiment the patients' reticulocyte counts were 3.8 per cent and 4.2 per cent.

Red Cell P1 and ATPase Activity

Inorganic phosphorus in 3 patients' washed red cells was higher than control levels (Table 2). The release of phosphorus from an ATP enriched aliquot of the same hemolysate during 1 hour of incubation also indicates that ATPase activity was increased in these patients' erythrocytes. The differences between patient and control values in both of these studies are highly significant.

Red Cell Na and K

Increased P1 and ATPase activity in washed erythrocytes from both Family A homozygotes was associated with significantly elevated levels of red cell Na. These patients' red cell K concentrations resembled control values.

Effect of ATPase Inhibitors upon Autohemolysis

Figure 3 shows mean and range values on autohemolysis studies performed on 10 controls, 2 PK deficient pairs of siblings from Family A and from Family B, and a patient who has hereditary spherocytosis. The first columns of each group show values on whole blood without additive. The other columns record the effect upon autohemolysis of adding 3 ATPase inhibitors in the sequential molar concentrations listed and also adding ATP (Family B) to the incubation systems.

ATPase inhibitors did not significantly increase autohemolysis in control or PK deficient blood when baseline values were within normal limits (Family A). The in vitro hemolysis of spherocytes was markedly increased by these agents, the extent of which hemolysis correlated with the concentration of inhibitor present. In contrast, all three ATPase inhibitors reduced autohemolysis of blood from both Family B homozygotes. The greatest effect was produced by p-chloromercuribenzoate; at $5 \times 10^{-5}$ M concentration this inhibitor proved as effective as ATP in correcting autohemolysis.

DISCUSSION

The variable clinical expression and the inheritance of red cell PK deficiency in our patients are in agreement with reported experience. It is evident
that inheritance of the underlying biochemical defect is not completely recessive. The heterozygous state can be readily detected by PK assay.

Only one patient has previously been reported with this condition whose red cell ATP was significantly elevated. The five patients in the present study had significantly higher levels of ATP in their red cells than normal control values. In vitro stability of ATP in these patients' red cells is at variance with reported experience. Absolute ATP levels are the product of synthesis and breakdown. Thus ATP decline during in vitro incubation of erythrocytes is not a true index of red cell ATP utilization. Increased ATPase activity does indirectly suggest ATP is broken down at an increased rate in our patients' red cells.

High energy phosphate requirements of normal erythrocytes are small. Apart from that needed for early glycolysis, ATP is mainly used to maintain an effective transmembrane transport of cations. Normal erythrocytes utilize only part of their glycolytic capabilities for the generation of ATP. This implies that red cells have built-in reserve capabilities for ATP synthesis. Production of ATP can be limited by diverting glycolysis through diphosphoglycerate cycles, thus bypassing the phosphoglycerate kinase reactions which generates ATP (Fig. 4). Depression of the diphosphoglycerate cycle, in the absence of increased demands for ATP, has been associated with an accumulation of ATP in red cells.

The generation of ATP in mature PK deficient erythrocytes must depend largely upon the phosphoglyceric kinase reaction. Increased red cell sodium content, as demonstrated in the present study, and increased bidirectional flux of potassium across the cell membrane indicate that erythrocyte membrane permeability to cations is increased in this condition. This places increased demands upon the sodium pump and ATP reserves which is reflected in increased ATPase activity and, in some instances, depletion of red cell ATP in vitro at an accelerated rate. There is no evidence, such as spherocytosis or...
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increased osmotic fragility, that the sodium pump is overburdened in PK deficient erythrocytes, as occurs in hereditary spherocytosis. These patients' PK deficient erythrocytes' ability to generate ATP appears able to compensate efficiently for increased demands. The extent to which the diversion of glycolysis from the diphosphoglycerate cycle through the ATP generating phosphoglyceric kinase reaction contributes to the maintenance of ATP levels in PK deficient erythrocytes remains to be determined.

Reports of ATP deficiency and instability indicate that adjustment of diphosphoglycerate metabolism may not be sufficient to maintain normal levels of ATP in PK deficient erythrocytes. This suggests a need for other compensatory mechanisms for ATP synthesis.

A net gain of ATP is possible by means of asynchronous glycolysis—that is, by a relative reduction of ATP utilization during early glycolysis. This can be achieved in a number of ways. A relative diversion of glycolysis through the hexose monophosphate pathway, which has been shown to occur in PK deficient erythrocytes, conserves ATP by avoiding the phosphofructokinase reaction, which requires high energy phosphate (Fig. 4). Alternatively, 1-glycerol phosphate or serine may be substituted for glucose as substrate for ATP synthesis. This obviates the need for ATP breakdown during early glycolysis. Thus, decreased glucose utilization by PK deficient erythrocytes would not necessarily be associated with a commensurate reduction in more distal glycolysis and ATP synthesis. A residual capacity for oxidative glycolysis augments ATP synthesis in PK deficient reticulocytes. This mechanism cannot have contributed significantly to ATP levels in 4 of our patients' red cells whose reticulocyte counts were not greatly elevated at the time of assay. Incubation with cyanide did not increase the rate of ATP depletion in red cells from 2 of our patients above control levels. This contrasts with Keitt's experience with a patient who had a marked reticulocytosis. The significance of increased red cell ATP in the present study is not clear. These values are undoubtedly influenced by the relatively young populations of red cells in the patients' blood. These ATP levels may, themselves, stimulate ATP synthesis in feedback fashion. Inorganic phosphate also stimulates ATP synthesis, even in PK deficient erythrocytes. Increased inorganic phosphate content may have contributed toward augmenting red cell ATP synthesis in the patients under study. Increased levels of ATP were probably not harmful to these patients' erythrocytes. Active transport of cations across erythrocyte membranes is accomplished by a number of complex, energy dependent, pump systems. These pumps derive most of their energy from ATP, which is broken down by sodium-potassium dependent, ouabain inhibited, ATPases that are present in red cell membranes. About 20 per cent of the energy required for transmembrane transfer of cations is derived from an electrolyte dependent, ethacrynic acid inhibited but not ouabain inhibited, pump. The source of energy for this pump is not ATP and is presently not known. Non-ATP dependent electrolyte transport systems has special interest in PK deficient erythrocytes.

Reduction of autohemolysis with different pharmacologic ATPase inhibitors adds further evidence that hemolysis of PK deficient erythrocytes in vitro does
not result from overburdened electrolyte transport systems. Studies on hereditary spherocytosis indicate these pumps have definite limitations to their work capabilities. Increased membrane permeability suggests that PK deficient erythrocytes also require increased pump activity like spherocytes. A critical inhibition of cation transfer, however, was not attained in the PK deficient cells.

The compensatory ability of these cells following pharmacologic inhibition of individual cation transport systems with different ATPase inhibitors was not evaluated. It is possible that sulfhydryl binding contributed to the correction of autohemolysis on blood from B-1 and B-2 with diisochloromercuribenzoate. However, reduction of in vitro hemolysis with ouabain and ethacrynic acid, which do not bind sulfhydryl bonds, suggests that inhibition of high energy phosphate release is the most important factor.

Incubation experiments with ATPase inhibitors suggest that in vitro hemolysis of PK deficient erythrocytes is associated with an ATPase dependent metabolic process. Reduction of metabolically available high energy phosphate with ATPase inhibitors slows glycolysis proximal to diphosphoglycerate, the glycolytic substrate for ATP synthesis. This effect reduces the accumulation of phosphoenolpyruvate and other products of glucose metabolism that may be toxic to the red cell. Increased autohemolysis has occasionally been observed when glucose is added to the incubation system. Decreased glucose consumption may be advantageous to PK deficient erythrocytes.

Increased ATPase activity and sodium content in these patients' erythrocytes probably reflect membrane hyperpermeability. Red cell membrane ATPase is sensitive to electrolyte changes and is stimulated by a relative increase in intracellular sodium. The concentrations of sodium and potassium within these patients' erythrocytes were almost maximal for sodium-potassium dependent ATPase activity. Increased red cell ATP content in these patients' erythrocytes may also stimulate ATPase by enzyme induction. Reticulocyte counts were not sufficiently elevated in the patients studied to have significantly influenced ATPase activity.

It is apparent that hemolysis of PK deficient erythrocytes can no longer be considered a direct effect of impaired ATP synthesis. The present study indicates that the basic cause for hemolysis of PK deficient erythrocytes remains to be determined. The degree of enzyme deficiency correlates poorly with clinical severity, autohemolysis and red cell ATP levels. This lack of correlation suggests the presence of factors that modify the effect of PK deficiency upon these parameters. The complex ramifications of glycolysis, even in the red cell, probably contribute to this situation. Caution is necessary in applying these in vitro observations to in vivo conditions.

**SUMMARY**

Elevated levels of ATP were observed in pyruvate kinase deficient red cells from 5 patients despite increased ATP requirements because of increased ATPase activity. Increased red cell ATPase activity was associated with an excess of intracellular sodium. Residual aerobic glycolysis in reticulocytes did not contribute significantly toward ATP generation in these patients' red cells. Their hemolytic anemia cannot be ascribed to a lack of high energy phosphate. Conversely, hemolysis in vitro appears to require a high energy phosphate dependent metabolic process.
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SUMMARIO IN INTERLINGUA

Elevate nivellos de ATP esseva observate in erythrocytos a carentia de kinase pyruvatic ab 5 patientes, in descpecto del facto que le augmentate activitate de ATPase habeva resultate in un augmento del consumo de ATP. Augmentos del activitate erythrocytic de ATPase esseva associate con un excesso de natrium intracellular. Un residuo de glycolyse aerobie in le reticulocytos non contribueva significativamente al generation de ATP in le erythrocytos de iste patientes. Lor anemia hemolytic non pote esser attrihuite a tin carentia de phosphato a alte energia. Inversemente, hemolyse in vitro pare requirer un processo metabolic que depende de phosphato a alte energia.

ACKNOWLEDGMENTS

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