Erythrocyte Pyruvate Kinase Deficiency in Non-spherocytic Hemolytic Anemia: A System of Multiple Genetic Markers?

By WOLF W. ZUELZER, ABNER R. ROBINSON AND THERESA H. J. HSU

ERYTHROCYTE PYRUVATE KINASE (PK) deficiency, described in 1961 by Valentine and his co-workers,1,2 is now considered the basic defect in most nonspherocytic hemolytic anemias not associated with an abnormal hexosemonophosphate shunt.3 The faulty behavior of the red cells in vivo and in vitro is ascribed to an energy deficit due to blocking of an ATP-producing glycolytic step and attended by malfunctioning of the cation pump.3,4 Reces-
sive inheritance is well documented, and PK assay is an accepted means of identifying homozygotes and heterozygotes for the abnormal gene,2,3,5 by implication a single mutant directly governing the synthesis of the enzyme.

Neither the biochemical nor the genetic scheme seems wholly satisfactory. The primary role of defective glycolysis in the hemolytic process has been questioned, notably by Busch,6 in view of the frequently normal glucose consumption of PK deficient cells. Nor does the block fully explain the in vitro instability of ATP observed in reticulocyte-rich populations of PK deficient erythrocytes,3,4 for the oxidative metabolism of immature red cells should make their ATP maintenance independent of anaerobic glycolysis.3,7 The genetic problem posed by the extreme variation in the severity of hemolysis from case to case remains unsolved. These phenotypic differences are seen within as well as between families and are unrelated to measurable differences in PK activity.6 Enzyme assays in heterozygotes also show inter- and intrafamilial variation2 and occasional marked overlapping with homozygotes, while conversely intermediate and even normal values have been reported in anemic patients presumed to be homozygous.8

The following study of two pedigrees exhibiting extreme intrafamilial pheno-
typic variation among severely PK-deficient subjects suggested that such seeming inconsistencies reflect genetic heterogeneity in a system in which the enzyme defect may serve as a genetic marker without necessarily constituting the ultimate cellular lesion. In contrast to the index cases characterized by excessive hemolysis, six clinically healthy relatives whose PK levels and genetic antecedents were equally compatible with homozygosity (and who could be differentiated from the numerous heterozygotes in these families) manifested minimal or no hematologic abnormalities. Genetic and biochemical findings were consistent with the existence of at least two allelic or pseudoallelic mutants whose interaction in various combinations may explain the differences between PK-deficient phenotypes and whose effect on PK activity may involve mechanisms other than direct control of the structure or synthesis of the enzyme.

METHODS

Standard hematologic procedures were employed. Autohemolysis was tested by the method of Selwyn and Dacie. Red cell survival was determined by the 51Cr technic described by Silver. PK was assayed by the procedure of Bücher and Pfeiderer as modified by Tanaka et al. Erythrocyte ATP, 2,3-diphosphoglycerate (2,3-DPG), d-3- and d-2-phosphoglycerate (3-PC and 2-PC), phosphoenolpyruvate (PEP), and pyruvate were measured according to the methods cited. Cells were washed and incubated in modified Krebs-Ringer solution at a phosphate concentration of 9.0 mM, according to the method of Mohler, and lactic acid* and glucose† were assayed in this laboratory according to stan-

*Obtained from Sigma Chemical Co., St. Louis, Mo. (Lactic Acid Kit 825).
†Glucostat, Worthington Biochemical Corp., Freehold, N. J.
Table 1.—Consecutive PK Values of Proposita (V-8) Following Transfusion of PK-deficient Erythrocytes from Mother and Aunt

<table>
<thead>
<tr>
<th>Days After Transfusion with Regular Donor's Blood</th>
<th>PK Values*</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Donor IV-4†</td>
<td>Donor IV-7†</td>
</tr>
<tr>
<td>42</td>
<td>0.413</td>
<td></td>
</tr>
<tr>
<td>61</td>
<td>0.458</td>
<td></td>
</tr>
<tr>
<td>75</td>
<td>0.45</td>
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</tbody>
</table>

*Expressed in terms of arbitrary units. One unit = that activity resulting in the conversion of one micromole of DPNH to DPN per minute by 10²⁰ leukocytes at 37 C. under assay conditions of these experiments.
†170 ml packed cells from IV-4.
1110 ml packed cells from IV-7.

Observations

Pedigree S

The proposita, V-8 (Fig. 1), was born with the clinical picture of hydrops fetalis, extreme pallor, edema, jaundice, and hepatosplenomegaly. The cord blood hemoglobin level was 6.0 Gm. per cent. Hemolytic disease and fetomaternal hemorrhage were ruled out by appropriate tests. The referring physician noted marked normoblastemia and distortion of the erythrocytes. The infant received an exchange transfusion and was discharged at 1 week with a hemoglobin level of 12.0 Gm. per cent, but returned at 1 month of age for further transfusions, the hemoglobin having fallen to 4.2 Gm. per cent. Normoblastemia was still evident, and the bone marrow showed marked erythroid hyperplasia.

Admitted to Children's Hospital of Michigan at 2 months, the patient was pale and exhibited splenomegaly. The hemoglobin (three weeks after the last transfusion) was 8.5 Gm. per cent, hematocrit 28.5 vol. per cent, RBC 3.48 × 10¹²/mm.³. Normoblastemia and reticulocytosis were still marked, but distorted red cells were no longer seen. Splenomegaly persisted, and continuing severe hemolysis necessitated transfusions at five to six weeks intervals throughout the five and a half years of observations to date. Direct and indirect antiglobulin tests were always negative, and except for anemia the patient's health and development were good.

Family studies established the tentative diagnosis of PK deficiency. Since the transfusion interval could not be extended to the point where normal donor cells would not interfere with PK assays, the patient was transfused successively with blood from her mother, IV-6, and her aunt, IV-7, both found to be severely PK-deficient. Serial assays thereafter showed a progressive decline to a value of 0.45 units seventy-five days after the last transfusion of normal donor blood (Table 1), indicating a degree of PK deficiency of the patient's own cells consistent with homozygosity. C-6-PD activity, assayed thirty-five days after an ordinary transfusion was normal.

*After completion of the above studies the patient underwent splenectomy at age 6. This was followed by the development of maximal reticulocytosis which has thus far enabled her to maintain hemoglobin levels of the order of 6 to 7 Gm. per cent without further transfusions. The reticulocyte counts which had not exceeded 10 per cent for several years began to rise about six weeks after splenectomy and reached a maximum of 90 per cent within four months. At this time, 125 days after the last transfusion, the PK assay showed a value of 0.71 units and C-6-PD activity was 34 units. The relatively high activity of both enzymes would appear to be related to the extreme reticulocytosis. The concentration of FEP was 10.2μM./100 ml. RBC, which is well within normal limits, but that of 2,3-DPG was markedly elevated, 1345 μM. (For interpretation of these latter findings see the discussion of the D. pedigree.) The rapid postsplenectomy development of reticulocytosis is noteworthy, as the supposition has been voiced that the reticulocyte count in severely affected patients continues to increase with age.³
Table 2.—Hematologic Data on Pedigree S.

<table>
<thead>
<tr>
<th>Pedigree S.</th>
<th>Hb Gm. %</th>
<th>HCT %</th>
<th>RBC (10^12/mm.³)</th>
<th>Cr¹ Survival (Half-Life, Days)</th>
<th>RET %</th>
<th>PK¹</th>
<th>%Hemolysis¹</th>
<th>24 hr.</th>
<th>48 hr.</th>
<th>ATP Added 48 hr.</th>
</tr>
</thead>
<tbody>
<tr>
<td>III-1</td>
<td>13.7</td>
<td>48.0</td>
<td>4.3</td>
<td>0.4</td>
<td>252²</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>III-6</td>
<td>11.2</td>
<td>39.0</td>
<td>4.3</td>
<td>2.8</td>
<td>1.3</td>
<td></td>
<td></td>
<td>0.91</td>
<td>3.90</td>
<td>0.71</td>
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<tr>
<td>IV-8</td>
<td>13.8</td>
<td>43.0</td>
<td>5.3</td>
<td>0.2</td>
<td>1.0</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>V-1</td>
<td>13.1</td>
<td>41.0</td>
<td>4.6</td>
<td>0.7</td>
<td>1.5</td>
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<tr>
<td>V-2</td>
<td>11.8</td>
<td>37.0</td>
<td>4.0</td>
<td>2.1</td>
<td>1.1</td>
<td></td>
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<td>1.7</td>
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<td>V-4</td>
<td>10.9</td>
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<td>5.3</td>
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<td>1.5</td>
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<td>V-5</td>
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<td>39.0</td>
<td>4.9</td>
<td>2.2</td>
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<td></td>
</tr>
<tr>
<td>V-6</td>
<td>10.8</td>
<td>36.0</td>
<td>4.3</td>
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<td></td>
<td>1.2</td>
<td>1.3</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>IV-1</td>
<td>13.2</td>
<td>42.5</td>
<td>4.5</td>
<td>5.7</td>
<td>0.1</td>
<td></td>
<td></td>
<td>0.58</td>
<td>5.20</td>
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<td>IV-4</td>
<td>12.3</td>
<td>39.0</td>
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<td>4.8-3.8</td>
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<td>0.62</td>
<td>1.54</td>
<td>0.69</td>
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<td>26-27</td>
<td>3.0-1.0</td>
<td>0.4</td>
<td></td>
<td>0.00</td>
<td>0.96</td>
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<tr>
<td>IV-7</td>
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<td>36.0</td>
<td>3.9</td>
<td>25-26</td>
<td>4.1</td>
<td>0.3</td>
<td></td>
<td>0.65</td>
<td>2.70</td>
<td>0.68</td>
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<tr>
<td>V-7</td>
<td>10.4</td>
<td>34.0</td>
<td>3.9</td>
<td>4.5</td>
<td>0.2</td>
<td></td>
<td></td>
<td>0.45</td>
<td>4.60</td>
<td>0.48</td>
</tr>
</tbody>
</table>

*Number of cases studied in this laboratory: Mean Range  
Normal 29 2.42 1.78-3.30 2.00-3.40  
Heterozygotes 23 1.19 0.61-1.78 0.73-1.73  
Tanaka’s Values²  
Normal values: 24 hr. 0.0-0.5; 48 hr., 0.4-3.5.

†Determination done by courtesy of Dr. Donald R. Harkness, University of Miami School of Medicine. The range of normal with this method is 300 to 500.

Nineteen relatives were studied. The family, of Syrian descent, was highly inbred, there being two cousin marriages, that of the parents and that of the paternal grandparents (Fig. 1). There was no history of anemia or jaundice on either side.

Ten subjects were identified as heterozygotes (Table 2). Nine of these, the brother, father, maternal grandmother, a maternal great-uncle, III-1, and five of six maternal cousins, V-1, V-2, V-4, and V-5 and V-6, all healthy and hematologically normal, showed intermediate values ranging from 0.99 to 1.50 PK units, in excellent agreement with those reported by Tanaka et al.² One maternal cousin, V-3, had borderline normal levels (1.69 and 1.78 units in separate assays) and, for reasons to be discussed, was also classified as heterozygous.

Five other relatives (Table 2), the sister, V-7, the mother, IV-6, and each of her three siblings, IV-1, IV-4, and IV-7, had PK values well within the range described for homozygotes,² extending from 0.07 to 0.39 units. Each of these subjects was healthy but showed borderline anemia, the hemoglobin levels ranging from 10.4 Gm. per cent (in V-7, a child) to 13.2 Gm. per cent, and the reticulocyte counts from 3.0 to 5.7 per cent. The 51Cr half life of the red cells, measured in IV-6 and IV-7, was twenty-six to twenty-seven and twenty-five to twenty-six days, respectively, compared to twenty-seven to thirty-five days for the controls. Autohemolysis was minimally increased in IV-1 and V-7, normal in the other three subjects. Glucose consumption and concentrations of pyruvate and its immediate precursors, PEP, 2-PG, and 3-PG, measured in the subjects listed in Table 3, were in the normal range. Lactate production was slightly decreased in IV-6, but in the range seen under identical conditions (four hours at high phosphate concentration) in the controls, in IV-7 and in V-7. The 2,3-DPG content, on the other hand, was approximately twice normal in the two subjects in whom it was assayed, and the ATP levels were about half those of the controls at the beginning of incubation with glucose but thereafter remained stable or even rose slightly. The activities of G-6-PD and of several glycolytic enzymes assayed were normal.

Comment. The five markedly PK-deficient relatives were clearly distinguish-
able from the heterozygotes in this pedigree by the stigmata of (albeit minimal) hemolysis and the decreased ATP and increased 2,3-DPG content of their red cells. There was, moreover, no overlapping of PK values between the two groups. Homozygosity for a "mild" PK deficiency gene, on the other hand, was not excluded by the genetic analysis based on PK assays. Of the three subjects in the group who had children, none had produced unequivocally normal offspring. The critical matings of the uncles, IV-1 and IV-4, with normal partners had yielded five obvious heterozygotes, and the borderline PK values of the sixth, V-3, were compatible with the presence of the trait. If the exceptionally low PK level of 0.07 units and the slight hemolysis observed in her father did indeed reflect the possession of two abnormal genes, this child had to be a carrier. The possibility that all four offspring of the maternal grandparents were homozygotes, although somewhat against Mendelian probability, was consistent with the consanguinity of this marriage in which the wife was a known carrier and the presence of an abnormal gene on the deceased husband's side was proved by the trait found in his brother, III-1.

Genetic analysis, however, must attempt to explain the extreme phenotypic difference between the severely anemic proposita and her markedly PK-deficient but virtually unaffected relatives with whom she must share at least one abnormal gene. If they were homozygous for the same mutant, it would follow that an identical genetic constitution could lead to maximal hemolysis in some subjects and a barely detectable disturbance in others. Although the frequent transfusions precluded detailed studies of red cell metabolism in V-8, there was no basis for assuming an additional genetic defect to account for her profound anemia. Conversely, the uniformity of the findings in the five healthy relatives excluded variation due to genetic modifiers as the cause of the exceptional mildness of their condition.

The dilemma would be resolved by the assumption of two distinct allelic or pseudoallelic mutants, each affecting PK activity in red cells. Double heterozygosity between interacting genes, as opposed to homozygosity for one of these genes, would account for the difference between the minimally affected relatives and the proposita. The PK levels of all subjects would fit into this scheme no less well than into that of a homozygous-heterozygous relationship for a single mutant, and while the notion that this highly inbred pedigree harbors two similar but separate mutations may seem awkward, it is through apparent discrepancies in the effects of genes brought together within a family that their differences often come to light. One need postulate only that I-1 and I-2, the great-great-grandparents (who had to be the original carriers), possessed different mutants.

This hypothesis does not exclude the existence of still other variants which might underlie the diversity of reported phenotypes. On the basis of only a two-gene model, and assuming that hemolysis of average severity corresponds to homozygosity for the commoner mutant, arbitrarily designated "A," the extreme hemolysis of the proposita would reflect the homozygous state for a rarer and more deleterious gene "B," while the minimal disturbance in
Table 3.—Biochemical Data* on Selected Subjects of Pedigree S

<table>
<thead>
<tr>
<th>Pedigree S.</th>
<th>Glycolytic Intermediates</th>
<th>ATP Stability</th>
<th>Glucose Consumed per hr.</th>
<th>Lactic Acid Produced per hr.</th>
<th>Lactate Glucose</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3-PG</td>
<td>2-PG</td>
<td>PEP</td>
<td>Pyruvate</td>
<td>2,3 DPG</td>
</tr>
<tr>
<td>IV-6</td>
<td>28.6</td>
<td>14.3</td>
<td>12.5</td>
<td>29.3</td>
<td>821</td>
</tr>
<tr>
<td>IV-7</td>
<td>23.8</td>
<td>19.8</td>
<td>17.8</td>
<td>37.8</td>
<td>834</td>
</tr>
<tr>
<td>V-7</td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

Normal Values:
- Range: 23.2-31.6
- Number Studied: (6) (8) (12) (12) (8) (11) (8) (8) (9) (9)

*All values expressed as mM/100 ml. RBC.
ERYTHROCYTE PYRUVATE KINASE DEFICIENCY

Her PK-deficient relatives would express the genotype AB. As discussed later, this does not imply, however, that these genes necessarily act through their effect on PK activity.

The near-normal blood picture of the five relatives permitted the study of PK deficiency in almost pure populations of mature red cells. The findings confirm Busch’s view that low PK activity in vitro does not necessarily mean impaired glycolysis in the intact cell, presumably because the normal erythrocyte possesses vastly greater amounts of the enzyme than required under in vivo conditions. Glucose consumption and pyruvate production were normal. None of the immediate proximal intermediates, PEP, 2-PG, or 3-PG, were increased. The low ATP levels thus could not be ascribed to a block in glycolysis, and their stability on incubation with glucose further militates against this mechanism. With glycolysis proceeding at a normal rate, the PK reaction, however deficient it might appear in vitro, should yield ATP, and the fraction thus derived apparently sufficed for ATP maintenance. Although mature, and thus unable to fall back on oxidative energy production, these cells were not “doomed by their enzyme defect,” as has been assumed for cases showing severe hemolysis. On the contrary, the low in vitro activity of PK was of little consequence for the survival of mature erythrocytes.

By the same token, however, the increase in 2,3-DPG, a metabolite several steps removed from the PK reaction, in mature cells with adequate glycolysis remains unexplained. In the absence of elevated 3-PG levels, it could not be attributed to inhibition of 2,3-DPG phosphatase by 3-PG as product. A primary disturbance in the 2,3-DPG cycle thus becomes a possibility. Such a defect in what has been called an “energy clutch” subsidiary to the main glycolytic pathway would not necessarily impair critical metabolic functions in the intact cell, and would thus be consistent with the minimal hemolysis in these PK-deficient subjects. Whatever the primary mechanism, an accumulation of 2,3-DPG should affect the yield of ATP from glycolysis, in keeping with the finding of low preincubation levels of ATP. The observation of an inverse relationship between 2,3-DPG and ATP in several red cell disorders has already caused speculation that anomalies in 2,3-DPG turnover may be of primary pathogenetic significance. How such a defect might depress PK activity is not clear, but a consideration of the biochemical pathways suggests that PK deficiency may be a secondary phenomenon rather than the basic abnormality, and as such governed by genes not directly concerned with the structural code for the enzyme.

Pedigree D

The proposita, III-3 (Fig. 2, Tables 4 and 5), briefly described in 1950 as a case of “unusual familial hemolytic anemia,” now 24 years old, was the third child of non-consanguineous Italian-American parents. Her two older brothers had died after birth with anemia and jaundice. She had survived a similar illness, misdiagnosed as erythroblastosis fetalis, had undergone splenectomy for continuing severe hemolysis at 4 months without apparent benefit, and had required transfusions with gradually decreasing frequency until the age of 18. Following cholecystectomy at age 15, she had remained well despite hemoglobin levels of 6 to 7 g/dl, had married, and held a clerical position. Except for extreme reticulocytosis (55 to 85 per cent), exhaustive studies had yielded
nothing of diagnostic import until 1963, when PK assays revealed deficiency of this enzyme. G-6-PD and other red cell enzymes assayed had normal or increased activities.

The youngest sister, III-5, had an almost identical course and laboratory findings, including erythrocyte PK values. She, too, had survived neonatal anemia and jaundice and had undergone splenectomy at age 3 with scant immediate benefit. Her transfusion needs had gradually declined with adolescence; she was then able to maintain hemoglobin levels of 6 to 7 Gm. per cent and was gainfully employed at age 22.

The erythrocytes of III-3 and III-5 showed marked autohemolysis, not corrected by addition of glucose or ATP. Despite the preponderance of reticulocytes, glucose consumption was low (Table 5), even when compared to that of mature normal cells. The concentrations of pyruvate, PEP, 2-PG, and 3-PG were in normal limits, but 2,3-DPG was greatly elevated. The initial ATP levels were in normal limits, but a precipitous fall occurred during preparations for incubation with glucose, especially in the cells of III-3 which lost three-quarters of their ATP in this period, while in those of III-5 the drop was less marked initially but continued to comparable levels during incubation.

Five members of the family* were identifiable as heterozygotes (Fig. 2, Table 4). Typical intermediate values were found in four of these: the mother, II-1, the paternal uncles, II-4 and II-5, and the middle sister, III-4. The PK levels of the father, II-2, was 0.78 units, lower than that of any of twenty heterozygotes studied in this laboratory but higher than that of his anemic daughters (or of the five presumptive double heterozygotes of the S. pedigree). Although in the range found in homozygotes by Tanaka et al.2 it was considered borderline, and the subject moreover could be identified as heterozygous on genetic grounds, his mother, I-3, being normal.

The paternal grandfather, I-4, on the other hand, although his hemoglobin level was above 15.0 Gm. per cent and his reticulocyte count was not elevated except on one occasion, did not appear to be a heterozygote. His PK level of 0.39 units, lower than that of the anemic granddaughters, was consistent with either homozygosity or double heterozygosity, as was the fact that his three living children by a normal wife were heterozygotes.

*Since completion of this study, an additional heterozygote, a healthy infant born to III-3, was studied and found to have a PK assay of 0.98 units.
ERYTHROCYTE PYRUVATE KINASE DEFICIENCY

Table 4.—Hematologic Data on Pedigrees D. and M.

<table>
<thead>
<tr>
<th>Pedigree D.</th>
<th></th>
<th></th>
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<th></th>
<th></th>
<th></th>
<th></th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Hb</td>
<td>HCT</td>
<td>RBC</td>
<td>RET</td>
<td>FK</td>
<td>24 hr.</td>
<td>48 hr.</td>
<td>ATP Added 48 hr.</td>
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<tr>
<td>I-3</td>
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<td>4.9</td>
<td>0.8</td>
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<tr>
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<td>6.3</td>
<td>30.8</td>
<td>34.1</td>
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Pedigree M.

Propositus 4.5-5.5 22 2.5 20.0-30.0 0.5 2.7 14.0 10.7

Mother 11.9 41 4.8 2.6 1.0

Father 14.9 44 4.3 1.4 0.9

Table 5.—Biochemical Data* on Selected Subjects of Pedigrees D. and M.

<table>
<thead>
<tr>
<th>Pedigree D.</th>
<th>Glycolytic Intermediates</th>
<th>Original ATP</th>
<th>ATP Stability</th>
<th>Glucose Consumed Per Hr.</th>
<th>Lactic Acid Produced Per Hr.</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-PG 2-PG PEP Pyruvate 2,3 DPG</td>
<td>0 hr. 2 hr. 4 hr.</td>
<td>0 hr. 2 hr. 4 hr.</td>
<td>0 hr. 2 hr. 4 hr.</td>
<td>0 hr. 2 hr. 4 hr.</td>
<td></td>
</tr>
<tr>
<td>III-3</td>
<td>14.8 21.0 30.7 132 36 29 36 93 112</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>III-5</td>
<td>13.0 20.5 28.7 1182 146 58 34 34 175 166</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I-4</td>
<td>8.3 3.2 30.1 510 86 82 73 82 139 272</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Pedigree M.

Propositus 88 17.4 40.8 50.1 640† 126 75 73 73 291 393

*All values expressed as μM./100 ml. RBC.
†Transfused seven weeks prior with 1,000 ml. packed cells.

in the case of the similar subjects in the S. pedigree, the second alternative was favored by the lack of overt hemolysis and by the contrast to severely anemic homozygotes within the family. Glucose consumption of his red cells was slightly decreased, the 2,3-DPG content was slightly increased, and the ATP level was slightly below normal and not restored by incubation with glucose (Table 5).

Comment. The findings in this family complement those in the S. pedigree. There, severe anemia was found only in one subject, the propositus. In the D. family, the apparently identical phenotype was represented by two sisters (and no doubt by their deceased brothers), and the similarity of their condition indicates that such excessive hemolysis is not, genetically speaking, accidental, but reflects the homozygous state for a highly deleterious gene. The pedigrees studied by McKusick et al. showed similar consistency of severe disease.

Conversely, the consistency of the findings in the minimally affected subjects of the S. family indicated a definite genetic constitution, presumably double heterozygosity, as the basis for a subclinical phenotype. Had not this clustering of low PK values in seemingly healthy subjects called for critical
scrutiny, the grandfather, I-4, of the D. pedigree, as an isolated case of PK deficiency without anemia, would have passed for a simple heterozygote whose low enzyme activity might be ascribed to variation, and the subtle abnormality in his red cell metabolism would have gone undetected.

It seems crucial that the red cells of the severely anemic sisters D., like those of the patients studied by Keitt and by Nathan et al. were mostly reticulocytes, i.e., cells for which a block in glycolysis should be of little consequence; yet in each case glucose consumption was low and ATP was very unstable, at least in vitro. Metabolically normal reticulocytes have a high glucose consumption which, as Keitt has shown, can be even further increased when the oxidative mechanisms available to young cells are blocked by cyanide, but which can also be maintained when anaerobic glycolysis is inhibited by fluoride. The inability of PK-deficient reticulocytes to utilize glucose and to maintain ATP would thus be consistent with an abnormality in both oxidative phosphorylation and (perhaps secondarily) glycolysis, for these cells behave much like normal reticulocytes poisoned with both cyanide and fluoride. The metabolic handicap of the young PK-deficient cells in the severely anemic subjects is thus as poorly explained by a block in glycolysis as is the lack of a comparable handicap in the mature erythrocytes of the minimally affected phenotype.

There is evidence for competition for phosphates between the oxidative and anaerobic energy systems of the reticulocyte. A primary abnormality seriously jeopardizing oxidative ATP synthesis (or making excessive demands on the ATP resources) would be likely to deprive the young cell of the reserves needed to carry on anaerobic glycolysis both before and after it matures. This would explain both the defective metabolism of the red cell of severely anemic PK-deficient subjects in the reticulocyte stage and its failure to survive as a mature erythrocyte. This implies, as in the subjects with minimal hemolysis, that the low activity of PK itself may represent an adaptation to a throttling of glycolysis. A possible clue in this direction may lie in Keitt’s observation that added phosphate doubled the glucose consumption of PK-deficient reticulocytes, which suggested to him that glycolysis was limited more by phosphate concentration than by PK.

**Propositus M**

This Negro male was severely jaundiced at birth and received an exchange transfusion although he did not have hemolytic disease. Anemia and splenomegaly were noted in infancy, and periodic transfusions were required. Untreated, the hemoglobin levels during ten years of observation were stable at 4.5 to 5.5 Gm. percent. The reticulocyte count was nearly constant at 30 per cent and was never less than 20 per cent (Table 4). PK deficiency was diagnosed after a suitable transfusion interval at age 7. Both parents showed intermediate PK values.

Glucose consumption of the child’s erythrocytes was in normal limits, although lower than expected for comparably reticulocyte-rich normal blood. ATP levels were approximately half normal but remained stable during incubation with glucose. The concentrations of PEP, 3-PG, and 2,3-DPG were all increased (Table 5).

**Comment.** Although hemolysis in this case was even more severe than in the anemic subjects of the D. family, the red cells showed better glucose
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consumption and ATP maintenance, probably reflecting the lower percentage of reticulocytes in the samples. This seemingly paradoxical explanation is suggested by the accumulation of PEP and 3-PG as proximal intermediates of pyruvate. The predominantly mature cells of M., having survived the postulated oxidative energy deficit during the reticulocyte stage, and relying on anaerobic glycolysis, are more apt to reflect the stress put on this system than are young cells less dependent upon this pathway. Allowing for the admixture of reticulocytes, the relatively high glucose consumption and the stability of ATP nonetheless indicate that glycolysis is by no means totally inhibited. In the case of D. sisters, the extreme reticulocytosis, evidently attributable to splenectomy,\(^{3,22,23}\) precluded detection of any impairment of glycolysis in mature cells through measurements of proximal intermediates.

**DISCUSSION**

PK deficiency appears to be the commonest metabolic abnormality found in the nonspherocytic hemolytic anemias formerly designated as “type II.” The extensive literature which has sprung up since its discovery in 1961 explicitly or implicitly embodies three closely related concepts: firstly, that the enzymatic defect, through its inhibitory effect on glycolysis, is the primary factor in the shortened survival of the red cells; secondly, that this defect results from mutation of a gene directly determining the protein structure (or possibly the rate of synthesis) of the enzyme; and thirdly, that PK assays reflect with reasonable accuracy a simple homozygous-heterozygous relationship. The observations given above are not readily reconcilable with the first two assumptions and call for modification of the third.

It is clear that while PK deficiency is a common denominator, the ultimate defect associated with extreme hemolysis and ATP instability in reticulocytes must differ either quantitatively or qualitatively from one compatible with near-normal survival and adequate ATP maintenance by mature erythrocytes. Indeed, to account for the severity exemplified here by the propositus S. and by the sisters D., some authors have invoked an additional membrane defect secondary to, or independent of, the glycolytic block.\(^{3,4}\) The familial pattern of the severe cases, however, makes the assumption of a separate defect untenable on genetic grounds, while the lack of serious derangement in the minimally affected subjects shows that a membrane lesion is not a necessary consequence of PK deficiency per se. The contrast between these phenotypes suggests that they represent different primary erythrocytic defects whose nature is unknown and whose effects on cell survival and in vitro behavior vary with the underlying genetic constitution.

This would explain the variability of biochemical and hematologic features observed in erythrocyte PK-deficient subjects. To some extent, however, these differences may be more apparent than real, for the findings in a given case of hemolytic anemia will be influenced not only by the genotype but by the age of the subject as it affects the rate of erythropoiesis, by the percentage of reticulocytes in the sample under study, and by the effect of splenectomy on the survival of these cells and thus indirectly on the metabolic profile of
the erythrocyte population. This is well illustrated by the comparison of the findings in the D. sisters with those on the propositus M., a case of comparable severity in which splenectomy was not performed.

Various metabolic defects related to glycolysis have been found in hereditary disorders, but their primary role in hemolysis is becoming increasingly doubtful. A case in point is in hereditary spherocytosis in which the defective cells survive normally after splenectomy. Keitt and Loder et al. concluded from their metabolic studies that glycolytic disturbances are not of primary pathogenetic significance in that condition. Busch has questioned the role of PK deficiency in nonspherocytic anemias, largely because of normal glucose consumption in the majority of cases, but most authors are agreed that defective glycolysis is at the root of these disorders. The theories proposed to explain apparent inconsistencies have been influenced by the assumption that the elevation of 2,3-DPG which has been called the hallmark of PK deficiency is wholly due to a glycolytic block downstream, the inference being that the directly proximal intermediates of pyruvate are also consistently increased, although their systematic determination is rarely done. The possibility that the high 2,3-DPG values might reflect a primary abnormality elsewhere has only recently been considered. Similarly, the apparent effectiveness, in terms of glucose consumption, of PK at low levels of in vitro activity in PK-deficient subjects has been attributed to the accumulation of substrate, PEP, yielding high reaction rates, but the findings in the PK-deficient relatives of the S. family show that mature erythrocytes can maintain adequate glucose metabolism at low enzyme levels in the absence of increased substrate.

The comparison of PK-deficient mature red cells in members of the S. pedigree with the almost pure reticulocyte populations of the D. sisters suggested that the elevation of 2,3-DPG in both types of subjects might be independent of the accumulation of glycolytic intermediates and of the rate of glucose consumption. The mature cells of the first group, moreover, were capable of maintaining ATP in vitro, while the reticulocytes of the severely anemic patients, despite the possession of a mitochondrial apparatus, were not. It seems conceivable therefore that a glycolytic block plays only a subordinate role in pathogenesis and that the decreased activity of PK may be a secondary response to unknown defects. These might involve diversion of phosphate into the 2,3-PDG cycle, but the abnormality in phosphate metabolism need not constitute the ultimate disturbance, for it might in turn hinge on a membrane defect making excessive demands on the energy resources of the maturing cell. A series of such defects of graded severity would explain the phenotypic differences seen in PK deficiency.

This line of reasoning, however, fails to explain the reduction of PK activity in the red cells of true heterozygotes which cannot be presumed to be related to any remote metabolic defect and which is in fact the strongest argument in favor of the primary role of PK deficiency in the anemia of homozygotes. Although the range of individual variation is so large that the influence of other factors has been suspected, the fact that the mean values of PK in heterozygotes are approximately half those of normals strongly suggests that the
mutant gene or genes act directly on the synthesis of the enzyme. Short of assuming that this process utilizes a common pathway (or requires identical building blocks) with an unidentified red cell component, i.e., postulating a dual effect for a single locus, the genetic patterns obtained through PK assays thus point to the enzyme defect as the ultimate biochemical lesion, regardless of imprecisions in quantitative regulation and of possible heterogeneity. The existence of multiple alleles or pseudoalleles would account for phenotypic differences, but the primary genetic substrate would be the same; since it is as difficult to explain the metabolic lesion in the reticulocytes of severely anemic patients solely as a glycolytic defect as to understand the survival of grossly PK-deficient mature erythrocytes in their relatives, the apparent conflict between genetic and pathophysiologic evidence remains unresolved.

The nature of gene action in the hemolytic anemias associated with PK deficiency thus remains in doubt. The basic role of the enzyme defect would be greatly strengthened by the demonstration of different isozymes of PK in some of the affected families, in analogy to the findings in the anemias associated with an abnormal hexose-monophosphate shunt. As yet no conclusive evidence of this sort is at hand. Valentine et al. and others found normal leukocyte PK activity in subjects whose red cells were deficient, and Koler et al. reported differences between normal leukocyte and erythrocyte PK in purified preparations, consistent with the existence of different structural genes in different tissues. It is conceivable, however, that the divergence of PK activity in these two cell lines in nonspherocytic anemia reflects not separate genetic control but differences in the metabolism of nucleated and denucleated cells; in other words, that the unique metabolism of the red cell may create special circumstances in which glycolytic and specifically PK activity would be depressed in the presence of an associated defect that would have no such effect in cells retaining their nucleus.

Attempts to characterize isozymes of PK in the heterozygotes of the above pedigrees were unsuccessful. In the framework of the classic theory of PK deficiency, it seems nevertheless probable that the subjects showing minimal hemolysis possess two different genes, one of which is responsible for a variant enzyme capable of functioning adequately in the intact cell in vivo despite the severe reduction of total in vitro activity. Unless such variants can be characterized through kinetic studies, the recognition of genotypes associated with PK deficiency remains tentative, and the distinction between homozygotes and double (or even simple) heterozygotes cannot rest on enzyme assays alone. For the present the rate of hemolysis and the systematic study of ATP stability, 2,3-DPG, and other intermediates, as well as glucose consumption of the red cells, allowing for the degree of reticulocytosis, would seem to be essential criteria for the genetic diagnosis of this group of nonspherocytic hemolytic anemias.

**SUMMARY**

Extreme intrafamilial differences between PK-deficient phenotypes regarding hemolysis, ATP stability, and glucose consumption were observed in two
pedigrees in which the index cases had severe nonspherocytic hemolytic anemia. Genetic analysis was consistent with heterozygosity for two distinct interacting mutants in minimally affected relatives of severely anemic homozygotes. Neither the mature erythrocytes of the former nor the reticulocyte-rich cell populations of the latter showed accumulation of glycolytic intermediates, but 2,3-DPG was elevated in both. Despite severe PK deficiency, red cell survival in the minimal type was near normal, glucose consumption was unaffected in three of four subjects tested, and ATP maintenance in vitro was adequate, in contrast to the severe type in which these parameters were grossly depressed.

The genetic and pathophysiologic implications of these findings are discussed. The possibility is considered that defective glycolysis may play a subordinate role in the hemolytic process associated with PK deficiency and that the enzyme defect may be a genetic marker for as yet unknown erythrocytic abnormalities involving an increase of 2,3-DPG and possibly primary membrane lesions creating excessive demands on the energy metabolism of the erythrocytes. Regardless of the mode of gene action, it is concluded that the nonspherocytic hemolytic anemias associated with PK deficiency are genetically and phenotypically heterogeneous, and that the genetic diagnosis cannot rest on PK assay alone.

SUMMARIO IN INTERLINGUA

Extreme differentias intrafamilial inter phenotypes a carentia de kinase pyruvatic esseva observate in duo studios familial suggestionate per le sever nonspherocytic anemia hemolytic del probandos. Le analyse genetic esseva compatibile con le these de heterozygositate pro duo distinte interagente mutantes in minimalmente afficite consanguineos de severmente anemic subjectos homozygotic. Ni le matur erythrocytos de illos, ni le populationes ric in reticulocytes de istos revelava un accumulation de intermediarios glycolytic, sed 2, 3-DPG esseva elevate in ambes. In despecto del sever carentia de kinase pyruvatic, le superviventia erythrocytic in le typo minimal esseva quasi normal, le consumption de glucosa esseva non-afficite in tres de quatro subjectos examineate, e le mantenentia de ATP in vitro esseva adequate, per contrasto con le typo sever in le qual ille parametros esseva marcamente deprimit.

Le signification genetic e pathophysiologic de iste constatationes es commentate. Es prendite in consideration le possibilitate que un glycolyse defective ha un rolo subordinate in le processo hemolytic associate con carentia de kinase pyruvatic e que le defecto enzymatic es forsan un indice genetic de un non ancora cognoscite anormalitate erythrocytic que comporta un augmento de 2, 3-DPG e possibilemente lesiones primari de membranas resultante in excessive demandas de energia ab le metabolismo del erythrocytos. Sia que sia le modo de action del gen, le conclusion pare justificate que le nonspherocytic anemias hemolytic associate con carentia de kinase pyruvatic es geneticamente e phenotypicamente heterogenee, de manera que le diagnose genetic non pote basar se exclusivemente in le essayage pro kinase pyruvatic.

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