Hereditary Hemolytic Disease Secondary to Glucose-6-Phosphate Dehydrogenase Deficiency: Report of Three Cases with Special Emphasis on ATP Metabolism

By Daniel N. Mohler and Charles L. Crockett, Jr.

Congenital nonspherocytic hemolytic anemia is a term that has been applied to patients suffering from a hereditary hemolytic process which cannot be attributed to hereditary spherocytosis, hereditary elliptocytosis, thalassemia, or one of the hemoglobinopathies. A better understanding of this heterogenous group of patients has been afforded in recent years as several specific red cell defects have been defined. Hereditary hemolytic disorders secondary to deficiencies in glucose-6-phosphate dehydrogenase (G-6-PD), pyruvate kinase, diphosphoglyceromutase, glutathione reductase, and reduced glutathione have been described. Because various red cell defects have been elucidated in these patients, it is preferable to discard the nonspecific term, “congenital nonspherocytic hemolytic anemia,” in favor of a specific nomenclature based on the underlying biochemical defect, i.e., hereditary hemolytic disease secondary to G-6-PD deficiency or pyruvate kinase deficiency or whatever deficiency has been demonstrated.

The present paper reports studies performed on three Caucasian men with hemolytic disease secondary to a deficiency in erythrocyte G-6-PD. Two of the patients are first cousins of Scotch-Irish-English descent and the other patient is of Turkish origin. Our studies on these patients show that a deficiency in G-6-PD leads to a fall in erythrocyte ATP under certain experimental conditions. The hemolytic process secondary to deficiencies in pyruvate kinase and diphosphoglyceromutase is also associated with a decrease in red cell ATP and suggests that a disorder in ATP metabolism may be a significant factor in the pathogenesis of several types of hereditary hemolytic disease.

Materials and Methods

Routine hematologic studies, hemoglobin electrophoresis, serum iron and iron binding protein, fecal urobilinogen and red cell osmotic fragility were performed by standard methods. Red cell survival times were determined by the use of sodium radiochromate.

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The following abbreviations are used throughout this paper: ATP, adenosine triphosphate; DPN, diphosphopyridine nucleotide; DPNH, reduced diphosphopyridine nucleotide; TPN, triphosphopyridine nucleotide; TPNH, reduced triphosphopyridine nucleotide; GSH, reduced glutathione; GSSG, oxidized glutathione; S.D., standard deviation.

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\( \text{(NaCr}_{51}\Omega_4) \) obtained from Squibb Laboratories. Autohemolysis was tested by incubating sterile heparinized whole blood and determining the amount of hemolysis present, as described by Jaffé. Glutathione stability was measured by the method of Beutler and the methemoglobin reduction test was performed as described by Brewer. G-6-PD was assayed by the method of Motulsky as described by Mohler and Williams. DPNH-dependent methemoglobin reductase was assayed by the method of Scott, as modified by Ross, except twice as much DPNH was used. Pyruvate kinase was assayed by the method of Tanaka, Valentine and Miwa. The conditions for the incubation of whole blood with phenylhydrazine and the chromatographic method used to measure erythrocyte ATP have been described in detail in a previous paper.

CASE REPORTS

Case 1

M. H., a 16 year old white student, was first noted to be anemic at age 2 when he was seen for a routine pediatric check-up. His hemoglobin at that time was 10.2 Gm. per cent and he was treated with ferrous sulfate for several years with no response. There was no history of neonatal jaundice. His anemia was mild until age 5 when he was hospitalized for what was thought to be "nephritis" and was treated with Elkosin following which his anemia became more severe but hemoglobinuria was not noted. His only other "hemolytic crisis" occurred at age 9 when he had an upper respiratory infection at the same time that his brother had measles. His hemoglobin fell to 4.5 Gm. per cent and he was admitted to the hospital where he received 2 units of blood. Since that time he has done well. The anemia has been mild with hematocrits varying from 36 to 39 per cent and reticulocytes from 3 to 8 per cent. His development has been normal with no limitation in activity. Physical examination has revealed no hepatomegaly, splenomegaly, or other abnormalities except for slight, occasionally scleral icterus.

Family history: The patient's maternal first cousin has a similar hemolytic anemia and is described as Case 2. A maternal uncle died at age 10 of an undiagnosed anemia which was accompanied by jaundice during the last 2 weeks of life. The patient's maternal grandmother was thought to be somewhat anemic for many years but there were no records available to confirm this. The pedigree of Cases 1 and 2 is shown in figure 1. None of the parents or siblings are anemic. The grandparents were of Scotch-Irish-English ancestry.

Laboratory examinations: In addition to the hematologic studies shown in table 1, the following laboratory examinations were performed: the white cell count was 6800 per cu. mm. with 44 per cent segmented cells, 46 per cent lymphocytes, 9 per cent monocytes, and 1 per cent eosinophils. The peripheral blood smear showed slight anisocytosis with moderate macrocytosis and polychromatophilia. There was no spherocytosis. Platelet count was 381,000 per cu. mm. Bone marrow was normal except for marked normoblastic erythroid hyperplasia. Fecal urobilinogen excretion was 501 mg. per day. Osmotic fragility determination after 24 hours' incubation at 37 C. was normal. Hemoglobin electrophoresis revealed AA hemoglobin and the patient's blood type was A, Rh positive. Serum electrophoresis showed a total protein of 7.6 Gm. per 100 ml. with 49.8 per cent albumin, 5.9 per cent alpha\(_1\) globulin, 10.0 per cent alpha\(_2\) globulin, 18.5 per cent beta globulin, and 17.8 per cent gamma globulin. The Wassermann test, Coombs test, tests for acid hemolysins, cold hemolysins, and cold agglutinins were negative. The results of tests of autohemolysis, glutathione stability, and methemoglobin reduction are shown in tables 2, 3, and 4 respectively. The results of the enzyme assays for G-6-PD, DPNH methemoglobin reductase, and pyruvate kinase are shown in table 5. The results of the ATP studies after incubation with phenylhydrazine are shown in table 6.

*These studies were performed according to the instructions supplied by Squibb Laboratories.
HEXEDITARY HEMOLYTIC DISEASE

HEREDITARY NONSpherOCYTIC HEMOLYTIC ANEMIA

DIED AT AGE IO YRS. OF UNKNOWN TYPE OF BLOOD DISORDER

* UNITS OF GLUCOSE-6-PHOSPHATE DEHYDROGENASE ACTIVITY

Fig. 1.—Pedigree of Case 1 (M. H.) and Case 2 (C. F.). The grandparents and maternal uncle are dead. Normal range for G-6-PD activity is 4.0-7.2 units (mean ±2 S.D.).

Case 2

C. F., the first cousin of Case 1, is a 21 year old white college student. He was first found to be anemic at age 6 when he was seen by a pediatrician for frequent episodes of tonsillitis. There was no history of neonatal jaundice. He was treated with iron and vitamin B-12 with no response and was referred to the University of Virginia Hospital at age 8 for further evaluation of his anemia. At that time his hematocrit was 35 per cent, reticulocyte count 16 per cent, and the bone marrow showed marked normoblastic erythroid hyperplasia. His spleen was palpated just below the left costal margin but has not been palpable since that time. Incubated osmotic fragility test was normal and no spherocytes were seen on peripheral blood smear. At the age of 10 the patient had a severe hemolytic crisis with jaundice, hemoglobinemia, hemoglobinuria and a fall in hematocrit to 22 per cent following an upper respiratory infection that was treated with Terramycin. On admission to the hospital the reticulocyte count was 1.4 per cent and it rose to 26 per cent during the recovery phase. At the age of 14 the patient had another acute episode of worsening of his anemia associated with pharyngitis and otitis media but with no hemoglobinemia or hemoglobinuria. This time his hematocrit fell to 15 per cent, reticulocytes were 0.2 per cent on admission and he received 4 units of whole blood. He has had no further hemolytic or aplastic crises and has been asymptomatic in respect to his anemia. His growth and development have been normal and there has been no limitation in activity. His hematocrit varies from 32 per cent to 40 per cent and in recent years has been 38 per cent to 40 per cent most of the time, with reticulocytes ranging from 3 to 8 per cent.

In addition to the laboratory studies listed in tables 1-6 and already mentioned above, other examinations disclosed the following: the white cell was 5800 per cu. mm., with 45 per cent segmented cells, 42 per cent lymphocytes, 3 per cent monocytes, 8 per cent eosinophils, and 2 per cent basophils. Platelet count was 496,000 per cu. mm. Hemoglobin electrophoresis showed AA hemoglobin and the blood type was O, Rh positive. Serum
Table 1.—Representative Hematologic Data in Three Patients with G-6-PD-deficient Hereditary Hemolytic Disease

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Case 1</th>
<th>Case 2</th>
<th>Case 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Packed red cell volume (%)</td>
<td>36</td>
<td>35</td>
<td>45</td>
</tr>
<tr>
<td>Hb. (Gm. %)</td>
<td>11.1</td>
<td>11.4</td>
<td>14.5</td>
</tr>
<tr>
<td>RBC (10⁶ per cu. mm.)</td>
<td>3.7</td>
<td>3.7</td>
<td>5.1</td>
</tr>
<tr>
<td>MCV (cu. μ)</td>
<td>97</td>
<td>94</td>
<td>90</td>
</tr>
<tr>
<td>MCH (μg.)</td>
<td>30</td>
<td>30</td>
<td>28</td>
</tr>
<tr>
<td>MCHC (%)</td>
<td>30</td>
<td>32.5</td>
<td>32</td>
</tr>
<tr>
<td>Reticulocytes (%)</td>
<td>7.8</td>
<td>8.2</td>
<td>3.6</td>
</tr>
<tr>
<td>Serum bilirubin (indirect/direct)</td>
<td>0.4/0.0</td>
<td>1.5/0.0</td>
<td>1.9/0.1</td>
</tr>
<tr>
<td>Serum iron/UBC* (μg. %)</td>
<td>146/175</td>
<td>135/270</td>
<td></td>
</tr>
<tr>
<td>Cr₅¹ RBC survival (T/2 days)</td>
<td>6.5</td>
<td>6</td>
<td>13</td>
</tr>
<tr>
<td>Bone marrow (M/E ratio)</td>
<td>1.2/1</td>
<td>.81/1</td>
<td>1.6/1</td>
</tr>
</tbody>
</table>

*Unsaturated iron binding capacity.

Table 2.—Results of Autohemolysis Studies in Three Patients with G-6-PD-deficient Hereditary Hemolytic Disease

<table>
<thead>
<tr>
<th>Subject</th>
<th>% Hemolysis after 24 Hours</th>
<th>% Hemolysis after 48 Hours</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Saline</td>
<td>Glucose*</td>
</tr>
<tr>
<td>Case 1</td>
<td>1.5</td>
<td>1.4</td>
</tr>
<tr>
<td>Case 2</td>
<td>1.8</td>
<td>2.2</td>
</tr>
<tr>
<td>Case 3</td>
<td>1.7</td>
<td>1.6</td>
</tr>
<tr>
<td>Normal range</td>
<td>1.0–1.9</td>
<td>0.7–1.7</td>
</tr>
<tr>
<td>Mean ± S.D.</td>
<td>1.5 ± 0.29</td>
<td>1.1 ± 0.33</td>
</tr>
</tbody>
</table>

*Glucose added in concentration of 40 μM/ml.

electrophoresis was normal. The serum Wassermann, Coombs test, tests for acid hemolysins, cold hemolysins and cold agglutinins were negative.

Case 3

S. C., a 38 year old Turkish physician, was first seen at the University of Virginia Hospital in August, 1961 for investigation of mild jaundice. He gave a history of having been jaundiced at the age of 16 for about 1 week at which time he was thought to have had hepatitis. However, there was not enough information available to determine whether he had liver disease or a hemolytic episode at that time. He did not notice jaundice again until approximately 18 months prior to being seen at the University of Virginia Hospital. When he noticed scleral icterus he checked his serum bilirubin several times and it varied from 2–3 mg. per cent, practically all of which was indirect reacting. He obtained several studies on his blood, including hematocrit, thymol turbidity, cephalin flocculation, and transaminases, which were normal. He had no symptoms other than generalized weakness and apprehension over his condition. There was no history of fever or exposure to anyone with hepatitis. He was not aware of jaundice again until 7 months prior to being seen. At that time he was not anemic and liver function tests were normal except for the bilirubin which was elevated and ranged from 2.0 to 4.5 mg. per cent with no elevation in the direct reacting fraction. Because of the persistence of the jaundice he came to the University of Virginia Hospital for further evaluation. He was asymptomatic except for some vague weakness. There was no family history of anemia.

The results of examinations other than those listed in tables 1–6 were as follows: white cell count 8600 with 70 per cent segmented cells, 25 per cent lymphocytes, 2 per cent monocytes, and 3 per cent eosinophils. No spherocytes were seen on the peripheral blood
Table 3.—Results of Glutathione Stability Test in Three Patients with G-6-PD-deficient Hereditary Hemolytic Disease, and in the Mothers of Cases 1 and 2

<table>
<thead>
<tr>
<th>Subject</th>
<th>Reduced-Glutathione mg. %</th>
<th>0 Hours</th>
<th>2 Hours with APH*</th>
<th>2 Hours with APH and glucose†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Case 1</td>
<td></td>
<td>31.8</td>
<td>5.9</td>
<td>7.7</td>
</tr>
<tr>
<td>Case 2</td>
<td></td>
<td>38.5</td>
<td>14.3</td>
<td>11.8</td>
</tr>
<tr>
<td>Case 3</td>
<td></td>
<td>43.3</td>
<td>12.0</td>
<td>13.8</td>
</tr>
<tr>
<td>Mother of Case 1</td>
<td></td>
<td>60.0</td>
<td>67.7</td>
<td></td>
</tr>
<tr>
<td>Mother of Case 2</td>
<td></td>
<td>53.6</td>
<td>52.5</td>
<td></td>
</tr>
<tr>
<td>Normal range</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(17 determinations)</td>
<td></td>
<td>50.0–94.4</td>
<td>&gt;41.9</td>
<td></td>
</tr>
<tr>
<td>Mean ± S.D.</td>
<td></td>
<td>66.6 ± 13.0</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Acetyphenylhydrazine 5 mg ml.
†Glucose added in concentration of 20 μM ml.

Table 4.—Results of Methemoglobin Reduction Test in Three Patients with G-6-PD-deficient Hereditary Hemolytic Disease and in Other Family Members

<table>
<thead>
<tr>
<th>Subject</th>
<th>% Methemoglobin Remaining*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Case 1</td>
<td>65.5†</td>
</tr>
<tr>
<td>Case 2</td>
<td>62.4†</td>
</tr>
<tr>
<td>Case 3</td>
<td>98.4</td>
</tr>
<tr>
<td>Mother of Case 1</td>
<td>6.2†</td>
</tr>
<tr>
<td>Mother of Case 2</td>
<td>10.1†</td>
</tr>
<tr>
<td>Father of Case 1</td>
<td>5.1</td>
</tr>
<tr>
<td>Brother of Case 1</td>
<td>4.5</td>
</tr>
<tr>
<td>Brother of Case 2</td>
<td>5.6</td>
</tr>
<tr>
<td>Father of Case 2</td>
<td>3.8</td>
</tr>
<tr>
<td>Sister of Case 1</td>
<td>5.0</td>
</tr>
<tr>
<td>Sister of Case 2</td>
<td>4.6</td>
</tr>
<tr>
<td>Sister of Case 2</td>
<td>5.1</td>
</tr>
<tr>
<td>Normal range (12 subjects)</td>
<td>3.8–6.7</td>
</tr>
<tr>
<td>Mean ± S.D.</td>
<td>5.1 ± 0.82</td>
</tr>
</tbody>
</table>

*Per cent methemoglobin remaining after 3 hours’ incubation of whole blood with sodium nitrite and methylene blue.
†Mean of three separate determinations.

I. Autohemolysis Studies

It can be seen from table 2 that the per cent of autohemolysis in the three
The niethemoglobin reduction test is an indirect measure of G-6-PD activity because, in the presence of methylene blue, methemoglobin is reduced primarily by a TPNH-dependent methemoglobin reductase and the generation of TPNH is impaired in the G-6-PD-deficient red cell.

### Table 5.—Results of G-6-PD, DPNH Methemoglobin Reductase, and Pyruvate Kinase Assays in Three Patients with G-6-PD-deficient Hereditary Hemolytic Disease

<table>
<thead>
<tr>
<th>Subject</th>
<th>G-6-PD*</th>
<th>DPNH Methemoglobin Reductase†</th>
<th>Pyruvate Kinase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Case 1</td>
<td>0</td>
<td>30.0</td>
<td>10.9</td>
</tr>
<tr>
<td>Case 2</td>
<td>0</td>
<td>30.5</td>
<td>9.6</td>
</tr>
<tr>
<td>Case 3</td>
<td>0</td>
<td>36.1</td>
<td>6.5</td>
</tr>
<tr>
<td>Normal range</td>
<td>4.6-7.4</td>
<td>29.1-36.7</td>
<td>3.4-5.9</td>
</tr>
<tr>
<td>Mean ± S.D.</td>
<td>5.6 ± 0.78</td>
<td>32.4 ± 2.64</td>
<td>4.4 ± 0.83</td>
</tr>
<tr>
<td>No. of normal subjects</td>
<td>35</td>
<td>12</td>
<td>10</td>
</tr>
</tbody>
</table>

*Expressed as change in O.D. min./ml. RBC.
†Expressed as change in O.D. min. × 10⁴.
‡Expressed as change in O.D. min., 10¹⁰ RBC.

### Table 6.—Summary of Studies Performed on Mothers of Cases 1 and 2

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Mother of Case 1</th>
<th>Mother of Case 2</th>
<th>Normal Range*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Packed red cell volume (%)</td>
<td>44</td>
<td>39</td>
<td>37–47</td>
</tr>
<tr>
<td>Reticulocytes (%)</td>
<td>1.4</td>
<td>1.8</td>
<td>0.5–1.5</td>
</tr>
<tr>
<td>GSH stability test</td>
<td>60.0</td>
<td>53.6</td>
<td>40.0–92.6†</td>
</tr>
<tr>
<td>2 hr. with APH</td>
<td>67.7</td>
<td>52.5</td>
<td>&gt;41.9</td>
</tr>
<tr>
<td>Methemoglobin reduction test (% methemoglobin remaining)</td>
<td>6.2</td>
<td>10.1</td>
<td>3.5–6.7†</td>
</tr>
<tr>
<td>DPNH methemoglobin reductase (Δ O.D. min. × 10⁴)</td>
<td>29.5</td>
<td>43.3</td>
<td>27.1–37.7†</td>
</tr>
<tr>
<td>G-6-PD (Δ O.D. min./ml. RBC)</td>
<td>4.4</td>
<td>4.1</td>
<td>4.0–7.2†</td>
</tr>
<tr>
<td>Cr¹¹ RBC survival (T/2 days)</td>
<td>23.5</td>
<td>23.0</td>
<td>28–35</td>
</tr>
</tbody>
</table>

*See tables 3, 4 and 5 for more details concerning normal values.
†Mean ± 2 S. D.
‡Acetylsphenylhydrazine 5 mg./ml.

patients was not significantly different than the normal controls at 24 hours and was only slightly greater than the control samples at 48 hours. The amount of autohemolysis was diminished by the addition of glucose.

2. Glutathione Stability

The results listed in table 3 show that the three patients had lower reduced glutathione (GSH) in their red cells than normal and had a marked fall in GSH after 2 hours' incubation with acetylsphenylhydrazine which was not prevented by the addition of glucose. The mothers of cases 1 and 2 had normal glutathione stability tests.

3. Methemoglobin Reduction Tests*

The per cent of methemoglobin remaining after 3 hours' incubation of whole blood with sodium nitrite and methylene blue is shown in table 4 for

*The methemoglobin reduction test is an indirect measure of G-6-PD activity because, in the presence of methylene blue, methemoglobin is reduced primarily by a TPNH-dependent methemoglobin reductase and the generation of TPNH is impaired in the G-6-PD-deficient red cell.
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the three patients and other members of the families of Cases 1 and 2. The
three patients had abnormal methemoglobin reduction under these circum-
cstances whereas all other family members were in the normal range except the
mother of Case 2 whose test was slightly higher than normal.

4. DPNH Methemoglobin Reductase Assays

The values for activity of DPNH methemoglobin reductase were within
the normal range for the three patients and the mother of Case 1, but were
somewhat elevated for the mother of Case 2. The values for the three patients
are shown in table 5 and for the two mothers in table 6.

5. G-6-PD Assays

No G-6-PD activity was detected in the red cells of the three patients. This
was the case even when a 1:50 hemolysate of whole blood was used in the
assay rather than the usual 1:250 dilution. G-6-PD activity in blood obtained
from the mothers of Cases 1 and 2 was at the lower limits of normal and lower
than any of the values of other members of the family, all of which were
in the normal range. The G-6-PD assay results are listed in table 5 for the
three patients, in table 6 for the two mothers, and in figure 1 for the whole
family.

6. Pyruvate Kinase Assays

Table 5 shows that the activity of erythrocyte pyruvate kinase was increased
above normal in the three patients. This increase in activity may be explained
by the reticulocytosis present since young red cells have greater pyruvate
kinase activity than do older cells.12

7. Family Studies

The family of Case 3 was not available for study. The parents and siblings
of Cases 1 and 2 were studied and no abnormalities were found except in the
mothers, who were studied in detail. The results of these studies are sum-
marized in table 6. The hematocrits, reticulocyte counts and glutathione sta-
bility tests were normal in both mothers. The methemoglobin reduction test
and DPNH methemoglobin reductase assay were normal in the mother of
Case 1 and abnormal in the mother of Case 2. Assays for G-6-PD activity were
at the lower limits of normal for both mothers, and red cell survival time by
the Crsup31 technic was slightly shorter than normal for both mothers.

8. Crsup31 Red Cell Survival Studies

The half-times of Crsup31-tagged red cells were 6½ and 6 days for cases 1
and 2 respectively, whereas it was 13 days for Case 3 (table 1). This corre-
lates well with the mild anemia in Cases 1 and 2 and the presence of no
anemia in Case 3. The Crsup31 half-times for the mothers of Cases 1 and 2 were
slightly shortened, being 23½ and 23 days respectively (table 6).

9. ATP Studies

Whole blood from the three patients was incubated for 8 hours at 37 C.
with and without added glucose, and phenylhydrazine and erythrocyte ATP
Table 7.—Erythrocyte ATP Metabolism in Three Patients with G-6-PD-deficient Hereditary Hemolytic Disease

<table>
<thead>
<tr>
<th>Case</th>
<th>ATP content prior to incubation</th>
<th>ATP content after 8 hours with the following additions:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ATP %ATP remaining</td>
<td>ATP %ATP remaining</td>
</tr>
<tr>
<td>Case 1</td>
<td>423.1 100</td>
<td>340.6 100</td>
</tr>
<tr>
<td>Case 2</td>
<td>317.6 75.1</td>
<td>290.5 83.3</td>
</tr>
<tr>
<td>Case 3</td>
<td>219.3 52.9</td>
<td>150.4 44.2</td>
</tr>
</tbody>
</table>

*μM/L. of whole blood.
†Twenty μM/ml. of glucose added.
‡Phenylhydrazine added in a concentration of 3.4 μM/ml.

The results listed in Table 7 show that there was a significant fall in ATP after 8 hours' incubation even when glucose was added, and a more pronounced fall in ATP in the presence of phenylhydrazine and in the absence of added glucose. Table 8 shows a comparison of erythrocyte ATP metabolism in normal subjects with normal erythrocyte G-6-PD activity, primaquine-sensitive Negroes with deficient erythrocyte G-6-PD activity, and the three Caucasian patients with no measurable erythrocyte G-6-PD activity. The results indicate that the amount of fall in ATP after incubation correlates with the severity of the G-6-PD deficiency, and that the greater the G-6-PD deficiency the less effective is glucose in preventing the fall.

DISCUSSION

Clinical Aspects and Review of Previous Reports

Hereditary hemolytic disease secondary to a deficiency in erythrocyte G-6-PD has been reported in 18 cases including the present report, and these are summarized in Table 9. Not included in this table are female relatives of some of these cases whose enzyme essays were in the intermediate range. Additional cases are mentioned in the literature in reports dealing with erythrocyte G-6-PD deficiency, but they are not included in the table because of inadequate clinical information.

The term hereditary hemolytic disease is used rather than hereditary hemolytic anemia because in some of these cases, such as the two Iranians reported by Waller and Löhrl and our case 3, the hemolytic process is well compensated and no anemia is present. In this regard the primaquine-sensitive Negro may be thought of as having a compensated hemolytic disorder since red cell survival has been shown to be shortened in the absence of administration of drugs. In a like manner, Caucasians from the Mediterranean littoral, who have hemolysis after ingestion of the Fava bean and who have a more marked red cell deficiency of G-6-PD than the Negro, may also have a mild compensated hemolytic process. However, the reports in the
Table 8.—Comparison of Erythrocyte ATP Metabolism in Normal Caucasians, G-6-PD-deficient Negroes and G-6-PD-deficient Caucasians

<table>
<thead>
<tr>
<th>Additions</th>
<th>% ATP Remaining after 8 Hours' Incubation, Considering ATP Content of Unincubated Samples as 100%</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Normal Caucasians*</td>
</tr>
<tr>
<td>Glucose</td>
<td>94.1</td>
</tr>
<tr>
<td>Saline</td>
<td>93.6</td>
</tr>
<tr>
<td>PH + glucose</td>
<td>88.5</td>
</tr>
<tr>
<td>PH + saline</td>
<td>35.1</td>
</tr>
<tr>
<td>Range of G-6-PD activity§</td>
<td>5.6–7.0</td>
</tr>
</tbody>
</table>

*The values given represent the mean of seven experiments on four normal Caucasians, seven experiments on four G-6-PD-deficient Negroes, and three experiments on three G-6-PD-deficient Caucasians. The individual data for the three G-6-PD-deficient Caucasians are listed in table 7.

§Twenty μM/ml. of glucose added.

§Phenylhydrazine added in a concentration of 3.4 μM/ml.

§G-6-PD activity expressed as change in optical density/min./ml. RBC.

literature dealing with this group of patients have been mainly concerned with the incidence and distribution of the red cell defect and with the acute hemolytic episode. There are no studies available at present in which the possibility of a mild chronic hemolytic disorder in these patients has been investigated.

We have included in table 9 those cases reported in the literature who had evidence of a chronic hemolytic disorder and in whose red cells G-6-PD activity was either absent or markedly decreased. Certain generalizations may be made about this group of patients. All of them were Caucasian males of either Northern European, British, or Mediterranean origin. The pattern of inheritance, when this could be determined, was sex-linked. The anemia was mild and accompanied by a moderate reticulocytosis. The majority of the patients had a history of neonatal jaundice and episodes of acute hemolysis and jaundice after infections or exposure to oxidant drugs. Although the G-6-PD-deficient Negro has acute hemolysis mainly after exposure to an oxidant drug, it was common for this group of G-6-PD-deficient Caucasians to have a severe hemolytic episode after a rather minor infection without exposure to any of the known offending drugs. This was true of our Cases 1 and 2. The spleen was enlarged in several of the children but in none of the adults. In those patients in whom a test for autohemolysis was done, the amount of spontaneous hemolysis after 48 hours' incubation was either normal or only slightly greater than normal and was improved by the addition of glucose, but to a lesser degree than normal. These findings place this group of patients into Selwyn and Dacie's Type I classification of congenital hemolytic anemia. Cr51 red cell survival studies were performed on six patients and the T/2 ranged from 5 to 13 days.

The total bilirubin was mildly elevated in half of the patients in spite of only a mild degree of hemolysis and there was no correlation between the degree of hemolysis and the degree of hyperbilirubinemia. Two of the patients with the mildest degree of hemolysis, Case 1 of Waller and Lohr and our
Case 3, had the highest bilirubins. Case 3, the Turkish physician, was referred to us because of intermittent jaundice and the possibility of constitutional hepatic dysfunction (Gilbert's disease). A hemolytic process had not been suspected because of his normal hematocrit and hemoglobin. When further study showed reticulocytosis and erythroid hyperplasia in the bone marrow, it became apparent that he had a hemolytic process and this was confirmed by demonstrating a shortened red cell survival. Conrad and his associates have pointed out that many patients who have been thought to have Gilbert's disease have had a hemolytic disorder that was not recognized because there was no anemia. In some of their patients the only indication of a hemolytic process was a reduced red cell survival time. Part of the explanation for the hyperbilirubinemia seen in patients with compensated hemolysis is the relatively large red cell mass affected. It should be emphasized that G-6-PD deficiency should be looked for in patients with unexplained jaundice and in patients with mild anemia. Although their anemia may be mild and asymptomatic, it is important that they be made aware of this enzyme abnormality so as to avoid exposure to offending drugs and thus preclude severe hemolytic episodes.

**Family Studies**

The family of Case 3 was not available for study. The pedigree of Case 1

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**Table 9.—Summary of Case Reports of G-6-PD-deficient Hereditary Hemolytic Disease**

<table>
<thead>
<tr>
<th>Author</th>
<th>Age (yrs.)</th>
<th>Nationality</th>
<th>PVC (%)</th>
<th>Hb (Gm. %)</th>
<th>Retic (%)</th>
<th>Total Bilirubin</th>
<th>Cr^T/2 (days)</th>
<th>Enlarged Spleen</th>
</tr>
</thead>
<tbody>
<tr>
<td>Waller et al.1,6</td>
<td>1. 50</td>
<td>Iranian</td>
<td>40</td>
<td>14.9</td>
<td>8.0</td>
<td>2.7</td>
<td>--</td>
<td>no</td>
</tr>
<tr>
<td></td>
<td>2. 41</td>
<td>Iranian</td>
<td>45</td>
<td>14.4</td>
<td>6.0</td>
<td>0.7</td>
<td>--</td>
<td>no</td>
</tr>
<tr>
<td>Newton et al.1,6</td>
<td>3. 4</td>
<td>Italian</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>5</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4. not given</td>
<td></td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5. not given</td>
<td></td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>Shahidi et al.1,6</td>
<td>6. 7</td>
<td>Italian, Polish</td>
<td>38</td>
<td>11.0</td>
<td>4.8</td>
<td>1.8</td>
<td>—</td>
<td>yes</td>
</tr>
<tr>
<td></td>
<td>7. 5</td>
<td>Italian, Polish</td>
<td>28</td>
<td>9.0</td>
<td>5.8</td>
<td>1.3</td>
<td>—</td>
<td>yes</td>
</tr>
<tr>
<td>Zinkham et al.1,6</td>
<td>8. 6</td>
<td>German, English</td>
<td>34</td>
<td>—</td>
<td>10.0</td>
<td>0.6</td>
<td>—</td>
<td>no</td>
</tr>
<tr>
<td></td>
<td>9. 2½</td>
<td>German, English</td>
<td>32</td>
<td>—</td>
<td>7.6</td>
<td>0.9</td>
<td>—</td>
<td>no</td>
</tr>
<tr>
<td></td>
<td>10. 56</td>
<td>German, English</td>
<td>55</td>
<td>—</td>
<td>14.3</td>
<td>—</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td></td>
<td>11. 10</td>
<td>German, English, French</td>
<td>34</td>
<td>11.2</td>
<td>10.8</td>
<td>2.2</td>
<td>—</td>
<td>no</td>
</tr>
<tr>
<td>Kirkman et al.1,6</td>
<td>12. 3</td>
<td>Italian, Dutch, English</td>
<td>—</td>
<td>10.7</td>
<td>11.0</td>
<td>—</td>
<td>splenectom</td>
<td></td>
</tr>
<tr>
<td></td>
<td>13. 2½</td>
<td>Italian, Dutch, English</td>
<td>30</td>
<td>9.9</td>
<td>8.6</td>
<td>1.3</td>
<td>—</td>
<td>yes</td>
</tr>
<tr>
<td>Nordoy et al.1,6</td>
<td>14. 37</td>
<td>Norwegian</td>
<td>—</td>
<td>12.3</td>
<td>8.5</td>
<td>1.9</td>
<td>7</td>
<td>no</td>
</tr>
<tr>
<td>Pansino et al.1,6</td>
<td>15. 1</td>
<td>Sardinian</td>
<td>32</td>
<td>9.8</td>
<td>7.7</td>
<td>1.2</td>
<td>5</td>
<td>yes</td>
</tr>
<tr>
<td>Mohler et al.1,6</td>
<td>16. 16</td>
<td>Scottish, Irish, English</td>
<td>36</td>
<td>11.1</td>
<td>3.7</td>
<td>0.4</td>
<td>6½</td>
<td>no</td>
</tr>
<tr>
<td></td>
<td>17. 21</td>
<td>Scottish, Irish, English</td>
<td>35</td>
<td>11.4</td>
<td>3.7</td>
<td>1.5</td>
<td>6</td>
<td>no</td>
</tr>
<tr>
<td></td>
<td>18. 38</td>
<td>Turkish</td>
<td>45</td>
<td>14.5</td>
<td>5.1</td>
<td>2.0</td>
<td>13</td>
<td>no</td>
</tr>
</tbody>
</table>

*All patients were males and the mode of inheritance when it could be determined was sex-linked. All patients had absent or markedly decreased erythrocyte G-6-PD activity.*
HEREDITARY HEMOLYTIC DISEASE

(M.H.) and Case 2 (C.F.) is shown in figure 1 and indicates a sex-linked mode of inheritance. The two patients are maternal first cousins, a maternal uncle died presumably of a severe hemolytic anemia at the age of 10 years, and the maternal grandmother was said to have had a mild anemia for many years, although she is now dead and no records were available to confirm this history. The two mothers who are presumably heterozygous for G-6-PD deficiency afford a good example of the difficulty that sometimes arises in demonstrating the enzyme deficiency in heterozygous females. The mother of Case 1 had a normal hematocrit, reticulocyte count, glutathione stability test, methemoglobin reduction test, DPNH methemoglobin reductase, and G-6-PD assay. The only hematologic abnormality demonstrated was a slight reduction in a Cr\textsuperscript{51} red cell survival half-time of 23.5 days. On the other hand, the mother of case 2 had a slightly abnormal reticulocyte count, methemoglobin reduction test, DPNH methemoglobin reductase assay and Cr\textsuperscript{51} red cell survival time. The values for hematocrit and G-6-PD assay were in the low normal range and the glutathione stability test was unequivocally normal. It should be pointed out that although none of our normal controls had G-6-PD values lower than 4.6 units, two standard deviations from the mean places the lower limit of normal at 4.0 units and includes the two mothers in the low normal range. In this regard all other family members who were studied were well within the normal range except for two of the sisters of Case 2 whose G-6-PD values were 4.8 and 4.5, which are at the lower limits of normal. They had normal hematocrits and methemoglobin reduction tests. It will be of interest in the future to study any children they might have to determine if they are carriers of the defective gene.

It has been pointed out previously that there is considerable variability in the degree of expressivity in females who are heterozygous for G-6-PD deficiency. Most of these women show intermediate values but a few are markedly affected and others show only slight or no abnormalities. Alving and his associates have pointed out that some women may have normal glutathione stability tests but show hemolysis in vivo after the administration of primaquine. Newton, in discussing the paper of Zinkham et al., mentions that the mother of one of his patients with G-6-PD deficiency had a shortened red cell survival time, although other tests were normal. Brewer and his co-workers have employed the methemoglobin reduction test as an index of G-6-PD deficiency and have found that approximately 80 per cent of known heterozygous females may be identified by this method which is similar to the results obtained with G-6-PD assays. In the mother of Case 1, only red cell survival studies pointed to a red cell abnormality, whereas in the mother of case 2, the methemoglobin reduction test as well as the red cell survival studies demonstrated an abnormality. It should also be pointed out that there was increased DPNH methemoglobin reductase activity in the red cells of the mother of Case 2. It is tempting to view this as a compensatory increase

*Beutler and his co-workers have proposed the X-chromosome mosaic theory to explain this variability in heterozygous females. According to this theory, only one of the X-chromosomes is genetically active and it is a matter of chance as to whether it is the normal or abnormal chromosome.
because of the impaired TPNH methemoglobin reductase activity shown by
the abnormal methemoglobin reduction test. However, in the three patients
who had no measurable G-6-PD and grossly abnormal methemoglobin reduc-
tion tests, the DPNH methemoglobin reductase assays were normal (table 5).

Mechanism of Hemolysis

Although it has been known since 1956 that the basic enzymatic defect in
the primaquine-sensitive erythrocyte is a deficiency of G-6-PD, the mecha-
nism by which a deficiency of this enzyme leads to hemolysis is still poorly
understood. A key feature in the G-6-PD-deficient erythrocyte is its abnormally
low GSH content which becomes lower after exposure to oxidant drugs. The
G-6-PD-deficient red cell is unable to maintain a normal GSH level be-
cause of its impaired ability to generate TPNH, a necessary cofactor for the
reduction of GSSG to GSH. The red cell with a reduced content of GSH is
handicapped in a number of ways. Jandl and his associates have empha-
sized the importance of membrane sulfhydryl groups in maintaining red cell
integrity. Mills and Randall have shown that GSH serves as a hydrogen
donor for red cell peroxidase and that this enzyme is capable of protecting
the red cell against oxidative injury. Cohen and Hochstein have demonstrated
that G-6-PD-deficient red cells are more sensitive to low levels of hydrogen
peroxide than are normal red cells and that oxidant drugs which produce
hemolysis in vivo can be shown to generate hydrogen peroxide in red cells.

These interrelated events may be summarized by the following chemical
equations:

1. Red cell + oxidant drug \rightarrow \text{H}_2\text{O}_2

2. \text{H}_2\text{O}_2 + 2 \text{GSH} \rightarrow 2 \text{H}_2\text{O} + \text{GSSG}

3. \text{GSSG} + \text{TPNH} + \text{H}^+ \rightarrow 2 \text{GSH} + \text{TPN}^+ \text{reductase}

4. G-6-P + TPN+ \rightarrow 6-

A deficiency in any of the three enzymes listed above would be expected to
lead to an accumulation of hydrogen peroxide and oxidative injury to the
red cell. Hemolytic disease secondary to a deficiency in G-6-PD is well known
and more recently hemolytic disorders secondary to a deficiency of glutathione
reductase and to an absence of GSH have been described. Hemolytic
disease associated with a deficiency of glutathione peroxidase has not as yet
been described.

The importance of glutathione peroxidase in protecting the red cell against
oxidative injury by hydrogen peroxide has been questioned because of the
large amount of catalase present in the red cell. However, Tarlov and

*See footnote on page 10.
†Glucose-6-phosphate.
‡Phosphogluconate.
Kellermeyer\textsuperscript{2} have shown that catalase activity is decreased in G-6-PD-deficient erythrocytes from primaquine-sensitive Negroes and falls further after drug-induced hemolysis.\textsuperscript{53} Also, Cohen and Hochstein\textsuperscript{54} have shown that low levels of hydrogen peroxide lead to a fall in GSH in the G-6-PD-deficient red cell but not in the normal red cell. Nevertheless, the role of glutathione peroxidase and catalase and their interrelationship in drug-induced hemolysis remains to be clarified.

In addition to the metabolic abnormalities mentioned above, Mohler and Williams\textsuperscript{24} and Löh and Waller\textsuperscript{54} have shown that the G-6-PD-deficient erythrocyte has an abnormal ATP metabolism. When G-6-PD-deficient red cells are incubated with phenylhydrazine\textsuperscript{24} or primaquine,\textsuperscript{54} there is a more pronounced fall in ATP than normal and there is less protection afforded by the addition of glucose than is the case with normal red cells. The present study shows a correlation between the severity of the G-6-PD deficiency and the extent of the ATP abnormality. When the three patients with no measurable erythrocyte G-6-PD were compared with normal subjects and with primaquine-sensitive Negroes whose red cells contained deficient but not absent G-6-PD activity, it was found that the fall in ATP after incubation with and without added glucose and with and without added phenylhydrazine was more marked in the three Caucasian patients than in the other two groups (table 8). In addition, there was a significant fall in ATP in the red cells of the three patients after 8 hours' incubation in the presence of adequate glucose and without the addition of phenylhydrazine. This was in contrast to the findings in normal subjects and in primaquine-sensitive Negroes, who showed no significant fall in red cell ATP under similar conditions. This correlates with the clinical observation that a more severe hemolytic disorder is present in Caucasians with G-6-PD deficiency than in primaquine-sensitive Negroes whose red cells show a lesser degree of G-6-PD deficiency. However, there are too few subjects in each group to make any valid comparisons between them at this time and only the trend in ATP metabolism may be noted.

Why a deficiency of G-6-PD should lead to an increased susceptibility to the ATP lowering effects of phenylhydrazine is another question to be answered. In a previous paper\textsuperscript{24} we showed that phenylhydrazine did not interfere with the synthesis of ATP but caused it to fall by increasing the rate of its utilization or destruction. Our present concept is that phenylhydrazine damages the red cell membrane both directly and through the production of hydrogen peroxide, which the G-6-PD-deficient red cell is poorly equipped to handle. The injured red cell membrane becomes more permeable to Na\textsuperscript{+} and K\textsuperscript{+},\textsuperscript{55,56} which in turn leads to increased activity of the Na\textsuperscript{+} — K\textsuperscript{+} pump in an effort to maintain osmotic equilibrium. Since the energy necessary for the Na\textsuperscript{+} — K\textsuperscript{+} pump is derived from ATP,\textsuperscript{57,58} one of the determinants of erythrocyte ATP content will be the balance between the rate of its utilization by the pump and the rate of its synthesis. If the red cell is subjected to glucose deprivation or increased pump activity exceeding the synthetic capacity of the cell, then the level of ATP would be expected to fall. When ATP falls below a critical level, the energy requirements of the red cell are not met and an increased rate of red cell destruction ensues. A decreased content of erythro-
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cyte ATP is associated with the impaired red cell survival that occurs with aging of red cells in vitro and in vivo and in the hemolytic anemia secondary to deficiencies in pyruvate kinase and diphosphoglyceromutase.

It should be evident from the preceding discussion that when a G-6-PD-deficient erythrocyte is exposed to an oxidant drug, a variety of metabolic injuries occur. It is probably not any one of these abnormalities but rather a combination of several of them (such as the disordered sulfhydryl and ATP metabolism) which leads to hemolysis. The fall in red cell ATP which occurs after incubation with oxidant drugs has been demonstrated only in vitro. Brewer and his associates observed no fall in the ATP of red cells from primaquine-sensitive Negroes who were given a hemolytic dose of primaquine. This may have been due to the selective destruction by the spleen of the older red cells with the lowest ATP values. Studies are currently in progress in our laboratory to investigate the role of ATP metabolism in drug-induced hemolysis in vivo.

Summary

1. Three cases of hereditary hemolytic disease secondary to G-6-PD deficiency are described. Two of the cases were first cousins of Scotch-Irish-English descent and the mode of inheritance was believed to be sex-linked. The third case was of Turkish origin; no family studies were available.

2. The mothers, who were heterozygous for G-6-PD deficiency, showed only minimal expression of the defect, which was manifested by a slightly decreased red cell survival in both mothers and an abnormal methemoglobin reduction test in one of them.

3. All three cases showed a more pronounced fall in erythrocyte ATP after incubation with phenylhydrazine than that observed in primaquine-sensitive Negroes whose red cells were less deficient in G-6-PD.

4. It is suggested that the inability of the G-6-PD-deficient erythrocyte to maintain adequate levels of ATP may be an important factor in the pathogenesis of the hemolytic process.

Summary in Interlingua

1. Es describite tres casos de hereditari morbo hemolytic secundari a carentia de dishydrogenase de glucosa-6-phosphato. Duo del patientes esseva cosinos del prime grado de ancestria scoto-iro-anglese, e le modo de transmission pareva esser sexualmente ligate. Le tertie patiente esseva de origine turc, e nulle studios familial esseva disponibile.

2. Le matres, heterozygotic pro carentia de dishydrogenase de glucosa-6-phosphato, manifestava solmente un minime expression de iste defecto: Ambes habeva levemente accurtate superviventias erythrocytic; in un, un anormal test de reduction de methemoglobina esseva obtenite.

3. Omne le tres patientes monstrava un plus pronunciate declino in le triphosphato adenosinic del erythrocytos post incubation con phenylhydrazina que primaquino-sensibile negros con erythrocytos minus carente in dishydrogenase de glucosa-6-phosphato.

4. Es postulate que in erythrocytos a carentia de dishydrogenase de glucosa-
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6-phosphato le incapacitate de mantener adequate nivellos de triphosphato de adenosina es possibilemente un importante factor in le pathogenese del processo hemolytic.

ACKNOWLEDGMENTS

We would like to thank Dr. Charles Behlen for performing the DPNH methemoglobin reductase assays, and Mr. Norman Eby for his technical assistance.

ADDENDUM

Since this manuscript was prepared for publication, Dr. Nuzet Atuk of the University of Virginia, Dept. of Internal Medicine, visited in Turkey and had the opportunity to perform the methemoglobin reduction test for G-6-PD deficiency on blood obtained from the mother and a brother of Case 3. Neither of them were anemic and in both the methemoglobin reduction test was normal.

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Hereditary Hemolytic Disease Secondary to Glucose-6-Phosphate Dehydrogenase Deficiency: Report of Three Cases with Special Emphasis on ATP Metabolism

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