Hemolytic Anemia Associated with Low Erythrocyte ATP

By Gordon C. Mills, William C. Levin and Jack B. Alperin

In 1964, Grimes, Meisler and Dacie described a patient with severe hemolytic anemia which they termed congenital Heinz body anemia. The red cells were characterized by Heinz bodies and a high percentage of reticulocytes (20 to 50 per cent). Biochemically, these cells exhibited a high glycolytic rate, but a low ATP level. Other investigators have also described patients with Heinz bodies in erythrocytes. These have been associated, in most cases, with mild hemolytic anemia. In some of these cases, an abnormal hemoglobin has been characterized (hemoglobin Köln). More recently, studies by Jacob et al. on a patient with Heinz body anemia with hemoglobin Köln indicate that Heinz bodies represent modified hemoglobin attached to the cell membrane. As a consequence of this attachment, the properties of the membrane in this case were altered. At the present time, it is not clear whether Heinz bodies in all patients with Heinz body anemia are due to an abnormal hemoglobin. For a more detailed discussion of unstable hemoglobins in hemolytic anemias, reference may be made to a review by Heller. We have observed a patient with erythrocyte inclusion bodies with biochemical characteristics of the erythrocytes similar to those noted in the patient of Grimes et al. A number of biochemical studies have been performed with these erythrocytes in an effort to elucidate the cause of hemolysis. A low red cell ATP level, despite a markedly increased glycolytic rate, and a markedly increased rate of breakdown of adenine nucleotides to hypoxanthine were the most prominent features of the erythrocytes. Other studies suggest that these unusual biochemical features are a consequence of excessive ATP utilization by the red cells. Enzymic assays and anion-exchange studies of phosphate esters provide strong evidence that the modifications in cellular metabolism are not due to a deficiency of glycolytic or pentose phosphate pathway enzymes. Other characteristics suggest that the
biochemical abnormalities may be a consequence of modifications in the erythrocyte membrane, although no direct evidence supporting this view is presented.

In recent years an increasing number of cases of hemolytic anemia involving an inherited abnormality of a specific protein of erythrocytes has been reported. In many of these, the abnormal protein is either hemoglobin or an enzyme. Erythrocytes have protein components other than hemoglobin and enzymes, and the structure of these proteins is also under genetic control. Although the genetic control of carbohydrate and lipid moieties of erythrocyte membranes is poorly understood, it is probable that these components also are determined by information contained in the DNA of erythrocyte precursor cells. Consequently, it is not surprising that many of the various inherited hemolytic anemias cannot be explained on the basis of an abnormality of hemoglobin or of enzymes. Structural proteins or nonprotein components of the erythrocyte membrane may also be subject to inherited modifications which may alter the properties and capabilities of the cell. Studies of hereditary spherocytosis have provided evidence for a membrane defect. In this case, the morphologic changes (sphering) and physiologic changes (increased passive sodium influx) may both be reflections of a basic membrane abnormality.

Alterations in metabolism of erythrocytes would be an expected consequence in most cases of membrane abnormalities, whether these abnormalities affect active transport or whether they affect rates of passive transfer of ions or other small molecules. The detection of alterations in metabolism, however, may require the application of more precise and sensitive analytic technics than those generally used. In some instances, these metabolic alterations might effectively compensate for the membrane abnormality and the lifespan of the cell might approach a normal value. In others, metabolic compensatory mechanisms might be relatively ineffective and the cell would pursue a progressive downhill metabolic course leading to early cell death. In addition to heritable membrane abnormalities, membrane damage may occur because of limitations in intracellular protective mechanisms, or as a consequence of the presence of unstable hemoglobin within the cell. Recent studies have indicated that membrane damage is a major consequence of drug administration to subjects with glucose-6-phosphate dehydrogenase (G6PD) deficiency, and the presence of an unstable hemoglobin (Köln) within the erythrocyte has also been reported to cause alterations of the membrane. Studies of the metabolism of erythrocytes with apparent membrane abnormalities, or membrane abnormalities produced as a consequence of treatment with drugs have been reported. Although no metabolic alteration has been noted in many cases, some investigators have found that cells did not maintain levels of ATP during in vitro incubation. It is likely that a failure in maintenance of ATP in vivo would lead to cell death. Possible metabolic consequences of membrane abnormalities have been emphasized in a recent review by Jandl.

**Materials and Methods**

**Materials**

5-Phospho-α-D-ribosyl-1-pyrophosphate (Mg salt) (PRPP) was purchased from either Sigma Chemical Co. or P-L Biochemicals. The purity of these preparations varied from 50 to
75 per cent. Lactic dehydrogenase (Type III, pyruvate-kinase-free), pyruvate kinase (Type II), oxidized and reduced nicotinamide adenine dinucleotide (NAD and NADH), oxidized and reduced nicotinamide adenine dinucleotide phosphate (NADP and NADPH), phosphoenolpyruvate (sodium salt), 2-phosphoglycerate (barium salt), glucose 6-phosphate (sodium salt), and oxidized glutathione (GSSG) were obtained from Sigma Chemical Co. Analytic grade ion-exchange resins (200/400 mesh) were obtained from Bio-Rad Laboratories.

Methods

Routine hematologic studies, hemoglobin electrophoresis, serum iron and iron-binding capacity, and red cell osmotic fragility were performed by standard methods. Autohemolysis studies were performed as described by Selwyn and Dacie, and red cell survival studies as described by Donohue et al.

Incubation of heparinized blood samples with shaking on a Dubnoff metabolic incubator at 37 C. was carried out under 5 per cent CO₂: 95 per cent N₂ as described previously. Sufficient 5.2 per cent glucose was added to raise the blood glucose level to approximately 200 mg./100 ml. of blood. In order to prevent alterations in levels of nucleotides or phosphate esters, all blood incubation studies were initiated within 20 min. after withdrawal of blood. When blood samples were not incubated, filtrates for anion-exchange analysis were prepared within 20 min. of the time of blood withdrawal. Carbon dioxide loss was prevented during this time by keeping blood samples in full closed tubes.

The erythrocytes were not chilled, nor were they washed to remove leukocytes in these studies, since both of these procedures may cause alterations in levels of nucleotides or other phosphate esters. The procedures for preparation of blood filtrates and determination of nucleotides and phosphate esters by anion-exchange analysis have also been described.

Hypoxanthine was determined in the column wash from the anion-exchange separation by a modification of a cation-exchange separation described previously. A 0.5 cm. by 20 cm. column of Dowex 50-X4 (H form) was used, and gradient elution was carried out by adding 1.5 N HCl dropwise to a mixing reservoir containing 75 ml. of water. Hypoxanthine and adenine are eluted after approximately 56 ml. and 140 ml. of eluent, respectively.

Enzyme assays using a Beckman DU spectrophotometer were carried out at 25 C. The temperature was maintained by circulating water from a constant temperature bath through Beckman thermospacers (No. 2075) placed in the instrument on both sides of the cuvet holder. Assay of the following enzymes was carried out by determining the rate of formation or utilization of NADH or NADPH from absorbance measurements on the spectrophotometer at 340 μm. The procedures, which were used with only minor modifications, follow: G6PD and 6-phosphogluconate dehydrogenase; GSSG reductase; and pyruvate kinase. Enolase was determined under the same conditions as the pyruvate kinase assay, with the addition of pyruvate kinase to the sample. However, 2-phosphoglycerate was utilized as substrate instead of phosphoenolpyruvate. Duplicate enzymatic assays were carried out in each case. For the pyruvate kinase and enolase assays, appropriate controls were carried out to eliminate the possibility of interference from contaminating enzymes, since commercial purified enzyme preparations are a required component of these assays. For the GSSG reductase assay, a small correction was made for the non-enzymatic oxidation of NADPH under the assay conditions.

As described by the original authors, these assay procedures utilize slightly different methods for preparation of hemolysates. In order to simplify this portion of the assays, the following method was utilized in our studies for the five enzyme assays described above. Two ml. of packed cells were added to 8 ml. of water. After standing for 40 min. at room temperature, the isotonicity of the sample was restored by addition of 2 ml. of 4.5 per cent NaCl. The stromal precipitate was removed by centrifugation. The stroma-free hemolysate was kept in an ice bath until used for the various enzymic assays. Activity of the various enzymes in this hemolysate appeared to be constant for at least four hours.

*Blood collections may be made in Vacutainer tubes if the entire tube is filled. In partially filled tubes, CO₂ loss may raise the blood pH and alter the relative amounts of ATP, ADP, and AMP.
Whole blood, in the presence of adequate glucose, may be kept at least three days at 4 C. with no loss of activity to these enzymes. Pipetting of packed cells from the middle of a centrifuged sample of heparinized blood provides a means of minimizing possible contamination of the sample by either leukocytes or plasma. Precise pipetting of cells in making the hemolysate is unnecessary because the amount of enzyme is ultimately expressed as umoles per hour per Cm. Hb. The hemoglobin is determined spectrophotometrically in the actual sample that is analyzed by measuring the absorbance of the sample at 542 μm after completing the enzymatic assay. An extinction coefficient (E1010) for oxy-hemoglobin of 8.92 at 542 μm was used in calculating the hemoglobin concentration in the analyzed sample. If necessary, the hemolysate preparation described above can be scaled down for use with smaller volumes of blood.

For the determination of adenosine triphosphatase (ATPase) activity, erythrocytes were hemolyzed by freezing and thawing three times. The whole hemolysate was used to determine the rate of liberation of inorganic phosphate from ATP in the presence of Mg, Na, and K ions as described by Harvald et al.26

Adenine phosphoribosyl transferase (EC 2.4.2.7) catalyzes the formation of adenosine monophosphate and pyrophosphate from adenine and PRPP. Adenine nucleotides present in the hemolysate would make the detection of newly formed adenine nucleotides more difficult. Consequently, the following procedure was used for the removal of most of the adenine nucleotides from the hemolysate. Stroma-free hemolysate, prepared by hypotonic lysis of cells as described for the above enzyme assays, was dialyzed for twenty hours against cold distilled water. The dialyzed hemolysate (ca. 12 ml.) was passed through a 1 by 8 cm. column of Dowex 1-X4, chloride form. KCl, 5.75 per cent* (0.25 ml. per ml. of hemolysate), was added to the effluent from the column to restore isotonicity of the sample. For the enzyme assay, each sample contained the following components: 0.10 M MgCl2, 0.15 ml; 1 mM PRPP, 1.50 ml.; 5 mM. adenine, 1.50 ml.; 0.10 M phosphate buffer, pH 7.4, 0.75 ml.; 0.15 M NaCl, 0.75 ml.; and hemolysate, 4.50 ml. Incubation was initiated by addition of the hemolysate and samples were incubated without shaking at 37 C. Four ml. aliquots of the samples were transferred to test tubes at zero time and after 30 min. of incubation. These test tubes were immediately placed in a boiling water bath for 10 min. to stop the reaction and deproteinize the samples. After addition of 8 ml. of water, the samples were mixed and centrifuged. AMP was determined in a 10 ml. aliquot of the supernatant fluid utilizing anion-exchange analysis with a multiple column technic. The AMP was eluted from the columns (Dowex 1-X4, formate form, 0.5 cm. by 14 cm.) with 0.2 N formic acid. A 0.50 ml. aliquot of the incubated samples was also utilized for the determination of hemoglobin, in order that enzyme activity might be expressed per gram of hemoglobin. Under the conditions of this assay, no AMP is converted to ADP or ATP. Recovery studies with AMP added to hemolysates indicated that no significant amounts of AMP were being degraded by the action of a nucleotidase or by adenylate deaminase. Recovery of added AMP averaged 94 per cent without incubation and 86 per cent after 60 min. of incubation.

Control Subjects

The younger cell population group (YCP) included in the data for Table 2, was composed of twenty-two hospital patients with elevated reticulocyte counts in response to either hemorrhage or hemolytic disease. These patients ranged in age from 3 months to

*Although 4.5 per cent NaCl was used for restoration of isotonicity in initial studies in this series, it was subsequently noted that activity was 36 per cent higher in the presence of potassium ions. Consequently, the values determined in the presence of NaCl have been multiplied by 1.36, so that all activities might reflect the higher value noted in the presence of potassium ions.

†AMP is stable under these conditions, and the use of heat to deproteinize samples permits the application of the filtrate directly to the columns. The small amount of protein remaining in the filtrate does not interfere with the subsequent AMP determination.
HEMOLYTIC ANEMIA

65 years with an average age of 31 years. The group included eleven Caucasians and eleven Negroes; it included five subjects with glucose-6-phosphate dehydrogenase deficiency, three with elliptocytosis, two with hereditary spherocytosis, and one with sickle cell anemia. Two subjects were bleeding and nine had hemolytic disease of unknown etiology. The twenty-six normal subjects included in the data presented in Table 2, ranged in age from 17 to 62 years, with an average age of 30. These subjects included twenty-three Caucasians, fifteen male and eight female, and three Negroes, two male and one female.

CASE REPORT

C.W. was born on February 3, 1952, following an uncomplicated labor and delivery. Her weight was 3.4 Kg. Hemolytic anemia was discovered at 10 months of age when she was examined for an upper respiratory infection. Splenectomy, performed eight months later, failed to alter the accelerated rate of erythrocyte destruction. The spleen weighed 129 Gm. Microscopic examination of the spleen revealed increased collagenous fibers about the sinusoids, follicular hyperplasia and blood filled pulp spaces and sinusoids. Reticulendothelial cells were filled with hemosiderin. Recurrent respiratory infections characterized her childhood. She found it difficult to do school work and could not always play with other children. One or more units of blood were transfused each year.

In November 1964, at age 12 years, she was admitted to the Clinical Study Center at the University of Texas Medical Branch for special hematologic and biochemical studies. She weighed 28.3 Kg. and was 137 cm. tall. Inspection of her head revealed the characteristic "facies" of hemolytic disease and icteric scleras. A loud, holosystolic ejection murmur was audible over the entire precordium, and the cardiac rate was 92 beats/min. Examination of the chest was normal. The edge of the liver was palpable 4 cm. below the costal margin and extended across the midline into the left upper quadrant.

The results of hematologic studies performed on this patient are listed in Table 1. The data indicate that the patient has severe hemolytic disease due to abnormal erythrocytes. These erythrocytes exhibited increased autohemolysis which was not corrected by the addition of glucose or ATP. The osmotic fragility of these cells was also increased. Examination of a Wright-stained blood film revealed macrocytic erythrocytes, target cells and spherocytes. Numerous erythrocytes were vacuolated. Large, dark-staining inclusion bodies were observed in some of the erythrocytes and are thought to present Pappenheimer bodies (Fig. 1). In reticulocyte preparations made with brilliant cresyl blue, inclusion bodies were visible in red cells when examined with an ordinary light microscope. These inclusions were present in approximately 20 per cent of the cells, and there was usually only one inclusion per red cell. Inclusion bodies were not observed in erythrocyte precursors, however. When blood smears were stained with Prussian blue, approximately 15 per cent of the red cells stained for iron.

The patient's erythrocytes did not sickle. Electrophoresis of a hemolysate on starch gel at pH 8.6 revealed four hemoglobins—A, A2, F, and an unknown component with a mobility in the general position of sickling hemoglobin.
Table 1.—Results of Hematologic Studies

<table>
<thead>
<tr>
<th></th>
<th>C. W.</th>
<th>Normal Values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hemoglobin Gm./100 ml.</td>
<td>8.5–10.5</td>
<td></td>
</tr>
<tr>
<td>Erythrocytes 10^9/mm.³</td>
<td>2.35–2.90</td>
<td></td>
</tr>
<tr>
<td>Hematocrit %</td>
<td>28–36</td>
<td></td>
</tr>
<tr>
<td>Reticulocytes %</td>
<td>35–67</td>
<td></td>
</tr>
<tr>
<td>Leukocytes /mm.³</td>
<td>15,400</td>
<td></td>
</tr>
<tr>
<td>Platelets /mm.³</td>
<td>453,000</td>
<td></td>
</tr>
<tr>
<td>Coombs test</td>
<td>negative</td>
<td></td>
</tr>
<tr>
<td>Sickle cell test</td>
<td>negative</td>
<td></td>
</tr>
<tr>
<td>Serum iron µg./100 ml.</td>
<td>236</td>
<td>75–135</td>
</tr>
<tr>
<td>Total iron binding capacity µg./100 ml.</td>
<td>368</td>
<td>300–375</td>
</tr>
<tr>
<td>Fecal urobilinogen excretion mg./24 hr.</td>
<td>622</td>
<td>200</td>
</tr>
<tr>
<td>Erythrocyte protoporphyrin µg./100 ml. cells</td>
<td>23</td>
<td>5–60</td>
</tr>
<tr>
<td>Erythrocyte glutathione mg./100 ml. cells</td>
<td>59</td>
<td>50–90</td>
</tr>
<tr>
<td>Autohemolysis %</td>
<td>7.5</td>
<td>0.4–4.5</td>
</tr>
<tr>
<td>+ glucose</td>
<td>8.6</td>
<td>0.3–0.7</td>
</tr>
<tr>
<td>+ ATP</td>
<td>4.1</td>
<td>0.0–0.8</td>
</tr>
<tr>
<td>Osmotic fragility (% NaCl)</td>
<td>0.6</td>
<td>0.5</td>
</tr>
<tr>
<td>Without incubation</td>
<td>0.3</td>
<td>0.3</td>
</tr>
<tr>
<td>With incubation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>beginning hemolysis</td>
<td>0.8</td>
<td>0.6</td>
</tr>
<tr>
<td>complete hemolysis</td>
<td>0.4</td>
<td>0.4</td>
</tr>
<tr>
<td>Quantitative hemoglobin analysis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>74%</td>
<td>97%</td>
</tr>
<tr>
<td>A₂</td>
<td>2%</td>
<td>2–3%</td>
</tr>
<tr>
<td>F</td>
<td>13%</td>
<td>1%</td>
</tr>
<tr>
<td>?</td>
<td>11%</td>
<td></td>
</tr>
<tr>
<td>^51Cr RBC survival T 1/2 days</td>
<td>4.0</td>
<td>25–30</td>
</tr>
</tbody>
</table>

Quantitative analysis of hemolysates indicated the presence of 74 per cent hemoglobin A, 12 per cent hemoglobin F, 3 per cent hemoglobin A₂ and 11 per cent of the unidentified component. Electrophoresis of the hemolysate after heating at 50 C. for three hours revealed loss of the unknown hemoglobin component; however, there was no significant reduction in the quantity of hemoglobins F and A₂. The nature of the unknown hemoglobin is currently under investigation.

A hemolysate, prepared from washed cells, was tested for the presence of a
thermolabile protein by incubation at 50 C. for two and a half hours as described by Grimes et al. A precipitate began to form after about 50 min. in the hemolysate from the patient's cells, and the amount of precipitate increased for several more hours. There was no decrease, however, in the total soluble heme proteins during incubation of the hemolysate. Total heme proteins were measured as carboxyhemoglobin after treatment of the samples with carbon monoxide and sodium dithionite. The protein precipitate which formed during the two and a half hour incubation period amounted to approximately 10 per cent of the total protein (based on ultraviolet light absorbance measurements at 280 mµ). The precipitate was washed twice with 0.15 M NaCl to yield a light tan colored product. It was dissolved in 1 N NaOH, the solution was treated with carbon monoxide and sodium dithionite, and the absorption spectrum of the solution was determined. There was a very low Soret band near 420 mµ accounting for only 0.2 per cent of the total heme proteins of the original hemolysate. Consequently, the precipitate contained no significant amounts of heme protein. There was no precipitate formation in any of five control hemolysates during two and a half hours of incubation at 50 C. Therefore, the hemolysate from the patient's cells contains a thermolabile nonheme protein. Whether the protein is globin, or whether it is related in any way to hemoglobin synthesis has not been determined at the present time. These findings contrast with those of Grimes et al, who found approximately 30 per cent of the hemoglobin from the erythrocytes of their patient to be heat labile.
Fig. 2.—Anion-exchange separation of phosphate esters of erythrocytes by gradient elution on Dowex 1-X4 columns. The solution in the upper reservoir initially was 1 N formic acid; it was changed to 4 N formic acid and to 4 N formic acid + 0.8 M ammonium formate at the times indicated by the arrows on the graph. The upper graph (W. H.) is an example of a normal elution profile after five hours of in vitro incubation for comparison with the elution profile of the patient (C. W., Exp. 3, five hour control sample). The separation on a cation-exchange column of hypoxanthine from filtrates of five hour incubated samples is shown in the graphs. Elution in this case was carried out as described in the text. Column size in each of the above separations was 0.5 cm. by 20 cm. and fractions of approximately 3.5 ml. were collected. The blood filtrates applied to the columns were equivalent to 0.80 ml. of W. H. cells and to 0.83 ml. of C. W. cells. Hx indicates hypoxanthine.

The amount of methemoglobin formed during the two and a half hour incubation of the patient’s hemolysate at 50 C. was not significantly different from that formed when normal hemolysates are incubated under these conditions. In addition, no appreciable amounts of methemoglobin were present in the patient’s erythrocytes in freshly drawn blood samples. Consequently, the hemoglobin of the patient’s cells is not unusually susceptible to oxidation. Other workers have noted an increased rate of methemoglobin formation in hemolysates from patients with Heinz body anemia.1,3

Total serum bilirubin was 2.4 mg./100 ml. of which 1.3 mg. was bilirubin glucuronide. Other tests of liver function were normal as were tests of renal function. An electrocardiogram and a chest roentgenogram were also normal. Plasma hemoglobin levels were not elevated, and there was no evidence of abnormal urinary pigments or hemoglobinuria. Skull roentgenograms showed changes typical of hemolytic disease since birth.
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Table 2.—Nucleotides and Phosphate Esters

<table>
<thead>
<tr>
<th>Component</th>
<th>C. W.*</th>
<th>C. W. Family (6)</th>
<th>YCP† (22)</th>
<th>Normal (26)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMP</td>
<td>3.0 ± 0.5</td>
<td>2.2 ± 0.6</td>
<td>3.4 ± 2.7</td>
<td>2.4 ± 0.07</td>
</tr>
<tr>
<td>ADP</td>
<td>22.5 ± 3.1</td>
<td>20.3 ± 1.9</td>
<td>21.6 ± 7.6</td>
<td>20.1 ± 2.9</td>
</tr>
<tr>
<td>ATP</td>
<td>104 ± 8</td>
<td>152 ± 16</td>
<td>168 ± 32</td>
<td>139 ± 17</td>
</tr>
<tr>
<td>ADP/ATP ratio</td>
<td>0.21 ± 0.04</td>
<td>0.14 ± 0.02</td>
<td>0.13 ± 0.06</td>
<td>0.15 ± 0.02</td>
</tr>
<tr>
<td>CDPX</td>
<td>9.0 ± 1.3</td>
<td>3.7 ± 0.2</td>
<td>7.5 ± 3.4</td>
<td>3.6 ± 1.2</td>
</tr>
<tr>
<td>NAD</td>
<td>8.6 ± 1.6</td>
<td>7.1 ± 0.7</td>
<td>8.3 ± 2.6</td>
<td>6.7 ± 2.1</td>
</tr>
<tr>
<td>NADP</td>
<td>6.8 ± 0.9</td>
<td>4.3 ± 0.4</td>
<td>5.5 ± 1.0</td>
<td>4.6 ± 0.8</td>
</tr>
<tr>
<td>GDP</td>
<td>4.5 ± 1.1</td>
<td>1.8 ± 0.9</td>
<td>2.7 ± 1.6</td>
<td>1.8 ± 0.8</td>
</tr>
<tr>
<td>GDPM</td>
<td>3.8 ± 0.2</td>
<td>1.7 ± 0.6</td>
<td>2.8 ± 1.9</td>
<td>1.7 ± 0.8</td>
</tr>
<tr>
<td>UDPG + UDPAG</td>
<td>9.6 ± 3.2</td>
<td>4.7 ± 2.4</td>
<td>7.1 ± 3.0</td>
<td>4.2 ± 1.2</td>
</tr>
<tr>
<td>GTP area</td>
<td>23.8 ± 2.0</td>
<td>10.0 ± 3.2</td>
<td>17.2 ± 7.5</td>
<td>10.8 ± 3.5</td>
</tr>
<tr>
<td>HDP</td>
<td>42.4 ± 11.8</td>
<td>27.6 ± 3.3</td>
<td>44.4 ± 18.5</td>
<td>31.4 ± 8.5</td>
</tr>
<tr>
<td>2,3-DPG</td>
<td>652 ± 60</td>
<td>512 ± 57</td>
<td>748 ± 123</td>
<td>521 ± 58</td>
</tr>
<tr>
<td>Inorganic P</td>
<td>203 ± 25</td>
<td>96 ± 16</td>
<td>124 ± 34</td>
<td>101 ± 21</td>
</tr>
<tr>
<td>Total organic P</td>
<td>1927 ± 155</td>
<td>1660 ± 114</td>
<td>2260 ± 286</td>
<td>1648 ± 135</td>
</tr>
<tr>
<td>AMP + ADP + ATP</td>
<td>131 ± 7</td>
<td>174 ± 17</td>
<td>197 ± 31</td>
<td>162 ± 18</td>
</tr>
</tbody>
</table>

*Standard deviations are based on four analyses carried out over a two year period.
†Values obtained with blood from patients with a younger cell population. This very diverse group was made up of individuals who were anemic or who showed a reticulocytosis.

Except for the inorganic P, which is expressed as µmoles per 100 ml of blood, values are expressed as µmoles per 100 ml of cells (mean values ± standard deviation). Abbreviations used which are not described in the text are: CDFX, cytidine and deoxycytidine diphosphate choline and cytidine and deoxycytidine diphosphate ethanolamine; GDP, GTP and GDPM, guanosine 5'-diphosphate, guanosine 5'-triphosphate, and guanosine diphosphate mannose; UDPG + UDPAG, uridine diphosphate glucose and uridine diphosphate N-acetylglucosamine; HDP, hexose diphosphate; and 2,3-DPG, 2,3-diphosphoglycerate.

Family Studies

The patient is of English, Irish, and Scotch ancestry. Her parents and four siblings are in good health. A fifth sibling died at age 6 months as a result of congenital heart disease. A maternal grandfather is the only living grandparent. The parents, the maternal grandfather, and all siblings have normal blood counts, normal erythrocyte morphology, and normal hemoglobin electrophoretic patterns. In addition, tests of osmotic fragility and autohemolysis performed on erythrocytes from both parents gave normal results.

Results

Nucleotide and Phosphate Ester Levels

The results of anion-exchange studies for nucleotides and phosphate esters in the patient's erythrocytes are shown in Figure 2 and Table 2. The values for these components are compared in Table 2 with values for these components (1) in normal erythrocytes, (2) in erythrocytes of patients with a younger cell population, and (3), in erythrocytes of six family members. Amounts of the following components in the patient's erythrocytes are elevated when compared with normal values: cytosine-containing nucleotides (CDPX), NAD, NADP, GDP mannose, UDP glucose + UDP N-acetylglucosamine, GDP,
Table 3.—Levels of Various Erythrocyte Enzymes

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>9/64</th>
<th>11/65</th>
<th>C. W. Family (6)</th>
<th>Normal Values (9)</th>
</tr>
</thead>
<tbody>
<tr>
<td>G6PD</td>
<td>485</td>
<td>442</td>
<td>250 ± 34</td>
<td>264 ± 37</td>
</tr>
<tr>
<td>6-Phosphogluconate dehydrogenase</td>
<td>384</td>
<td>336</td>
<td>229 ± 29</td>
<td>241 ± 23</td>
</tr>
<tr>
<td>Glutathione reductase</td>
<td>368</td>
<td>465</td>
<td>186 ± 37</td>
<td>198 ± 34</td>
</tr>
<tr>
<td>Pyruvate kinase</td>
<td>625</td>
<td>568</td>
<td>221 ± 60</td>
<td>382 ± 224</td>
</tr>
<tr>
<td>Enolase</td>
<td>—</td>
<td>700</td>
<td>410 ± 98</td>
<td>287 ± 57</td>
</tr>
<tr>
<td>Adenine phosphoribosyl transferase</td>
<td>—</td>
<td>14.4</td>
<td>8.2 ± 0.8</td>
<td>8.7 ± 2.4</td>
</tr>
</tbody>
</table>

All values are expressed in units of μmoles/hour/Gm. Hb (mean values ± standard deviation). If the activity of the enzymes in the C. W. erythrocytes were expressed per ml. of cells, the values would be 77 per cent as high when compared to normal values.

GTP, 2,3-diphosphoglycerate (2, 3-DPG), and HDP. When compared with values for subjects with younger cell populations, values for the above components probably are not elevated, but instead are typical of blood samples with an increased population of young red cells. In contrast, values for ATP and for total adenine nucleotides in the patient's cells are low when compared with erythrocytes of normal individuals and are markedly lowered when compared with values for ATP in blood with younger erythrocyte populations. The low value for ATP was noted on four occasions in blood samples studied over a time period of approximately two years. In addition, the ADP:ATP ratio was consistently higher (0.21±0.04) in the patient's cells than in cells from normal individuals (average, 0.15±0.03). The differences in adenine nucleotides which have been noted are more impressive when a comparison is made with other members of the patient's family, all of whom exhibit normal amounts of the various nucleotides and phosphate esters.

Another characteristic noted repeatedly in the patient's blood was the high level of inorganic phosphate (Table 2). The plasma phosphate level was only slightly above normal, but the level of inorganic phosphate within the cells was markedly elevated (269-301 μmoles/100 ml. cells). It is of interest to note that high levels of inorganic phosphate stimulate erythrocyte glycolysis. Consequently, the ionic environment within the cells is conducive to maximal hexokinase activity.

Another unusual characteristic of the patient's blood should be noted, even though its significance cannot presently be assessed. An ultraviolet-absorbing component was repeatedly observed in the elution profile immediately preceding NADP (Fig. 2). This component shows relatively nonspecific ultraviolet absorbance over the range of 260 to 300 mμ. It is not a nucleotide or other phosphate ester, and it is not pyruvic acid. Since all ion-exchange separations have been carried out with whole blood as a starting material, the compound may be a component of either plasma or erythrocytes. In numerous studies of blood from normal individuals, no similar component has been noted. The possibility that this component is a peptide has not been adequately investigated at the present time.
Erythrocyte Enzyme Assays

A summary of results of enzyme assays is presented in Table 3. Values for enzyme levels in the patient's erythrocytes are invariably higher than normal. Presumably, these high values are a reflection of a young cell population and do not indicate a basic enzyme abnormality. In view of the low ATP level, it is particularly significant that the primary enzyme involved in adenine nucleotide synthesis, adenine phosphoribosyl transferase, is also elevated in the patient's cells. Consequently, the low ATP level is not due to an enzyme deficiency in the adenine nucleotide biosynthetic pathway. Enzyme studies of erythrocytes of the patients' family were also normal. The only possible deviation from normal was the GSSG reductase assay of erythrocytes from the patient's mother, with values being obtained on two occasions in the low normal range. The reduced glutathione (GSH) level in this subject was elevated (107 mg./100 ml. cells). No particular significance is attached to these minimal variations, since they are not related to abnormalities in the patient's cells.

In addition to the enzyme studies shown in Table 3, erythrocyte ATPase activity in the patient's cells was approximately twice that noted in hemolysates prepared in the same manner from two normal individuals. This assay was carried out to eliminate the possibility of an ATPase deficiency as described by Harvald et al. This procedure does not provide an adequate measure of Na+, K+-activated ATPase activity in the erythrocyte membrane.

In Vitro Incubation Studies

Studies of the patient's cells following in vitro incubation at 37 C. are summarized in Figure 3 and Table 4. Three different series of studies were performed during a period of two years. The in vitro studies are complicated by the very high glycolytic rate. In the first experiment (Fig. 3A), the glucose level was depleted during incubation to a level at which glucose utilization was probably limited. In the second experiment (Fig. 3B), the supply of glucose was adequate, but the high rate of lactic acid production by these cells caused a larger fall in pH of the medium than occurs when normal cells are incubated. In the third experiment (Fig. 3C), the pH of the medium was maintained in the physiologic range by addition of small amounts of isotonic pH 8.8 tris buffer. Consequently, this last study was the most physiologic in vitro incubation study.

The primary abnormality noted in these studies is the high rate of breakdown of adenine nucleotides to hypoxanthine (4.3 μmoles/100 ml. cells/hour in Exps. 2 and 3). This value may be compared to a normal value of 1.3 ± 0.6 μmoles/100 ml. cells/hour, when cells are incubated in a 95 per cent N2:5 per cent CO2 atmosphere. Since ATP is the major adenine nucleotide, the nucleotide breakdown causes a marked fall in amount of ATP. It should be emphasized that this fall occurs in the presence of a glycolytic rate (7.6 μmoles glucose utilized/ml. cells/hour in Exp. 3) which is approximately three times the glycolytic rate of normal erythrocytes. In addition, the ADP:ATP ratio (Exp. 2, 0.32; Exp. 3, 0.19) remains higher following incubation for five hours than the corresponding value in normal cells (0.15). When adenine was added to
A. Exp. 1, study of Nov., '64: the sample was incubated five hours in air in a closed container; hematocrit, 34.7 per cent; glucose concentration: initial, 199 mg. per cent; final, 19.5 mg. per cent. B. Exp. 2, study of Nov., '65: the samples were incubated five hours under 5 per cent CO2 to 95 per cent N2; hematocrit, 36.5 per cent; glucose concentration: initial, 391 mg. per cent; final, 133 mg. per cent (control) and 146 mg. per cent (adenine). Adenine was added to the second sample prior to incubation at a concentration of 0.25 mg./ml. blood. C. Exp. 3, study of July, '66: the samples were incubated under 5 per cent CO2 to 95 per cent N2, with the pH maintained between 7.2 and 7.4 (measured at 37 C.) by addition of small amounts of isotonic pH 8.8 tris buffer; hematocrit, 33.6 per cent; glucose concentration: initial, 399 mg. per cent; final, 146 mg. per cent (control), and 128 mg. per cent (adenine). Adenine was added to the second sample prior to incubation at a concentration of 0.35 mg./ml. blood.

the patient’s cells prior to incubation, there was an increase in total adenine nucleotides (average, 9.3 per cent) during the five hour period. This increase was apparently the result of a substantial increase in rate of adenine nucleotide synthesis. A considerable amount of hypoxanthine was still produced under these circumstances, suggesting that the rate of adenine nucleotide breakdown was not decreased. Some of the hypoxanthine which was produced in this case is probably formed directly from adenine by adenine deaminase. The failure of adenine to alter significantly the ADP:ATP ratio of the cells during incubation is in accord with the view that adenine increases the rate of adenine nucleotide synthesis but has no significant effect on erythrocyte glycolysis.

The incubation studies also indicate that nucleotides in the GTP area of the elution profile are unstable. Since initial values were considerably above normal, the supply of these components within the cell did not fall below normal during a five hour incubation period. 2,3-DPG was also rather unstable in these
HEMOLYTIC ANEMIA

Table 4.—5 Hour In Vitro Incubation Studies

<table>
<thead>
<tr>
<th></th>
<th>C. W.</th>
<th>Normals and Patients (15)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycolysis rate</td>
<td>6.6</td>
<td>1.1–2.8</td>
</tr>
<tr>
<td>Hypoxanthine formation</td>
<td>4.3</td>
<td>1.3 ± 0.6</td>
</tr>
<tr>
<td>% of total adenine nucleotides in 5 hours</td>
<td>18–20</td>
<td>3.7 ± 1.9</td>
</tr>
</tbody>
</table>

cells in the first and second experiments (Figs. 3A and 3B). This may reflect, however, a greater fall than normal in the pH of the cells as a consequence of the high glycolytic rate. When the pH was maintained within the normal range (Fig. 3C), the level of 2,3-DPG fell only slightly during the incubation period. Although ATP in normal cells is stable at pH values slightly below the normal range, 2,3-DPG becomes progressively more unstable when the pH falls as low as 7.1.

DISCUSSION

Recent studies have shown that nonspherocytic hemolytic anemia may be due to a variety of biochemical defects of erythrocytes. Enzyme assays indicate that the patient described in our studies does not have a deficiency of G6PD, pyruvate kinase, glutathione reductase,28,29 or adenosine triphosphatase.26 Since hexokinase is one of the rate-limiting enzymes of the glycolytic pathway and erythrocyte glucose utilization was markedly above normal in our patient, there is no reason to suspect hexokinase deficiency.30 Prins et al.31 have described a family with a nearly complete deficiency of GSH in erythrocytes. The erythrocyte GSH level in our patient, although in the low normal range, does not suggest an abnormality in the formation of GSH. Frankerd and Bowdler2 have described a patient with an apparent deficiency of diphosphoglycerate mutase. As a consequence of this deficiency, these workers noted low levels of 2,3-DPG. Since levels of 2,3-DPG were slightly elevated in our patient, there is no deficiency of diphosphoglycerate mutase in her erythrocytes. Valentine et al.33 have recently described several cases of hemolytic anemia with a deficiency of triose phosphate isomerase. Although specific assays for this enzyme were not performed with our patient, neither the clinical characteristics of the anemia nor the results of the anion-exchange analysis suggest triose phosphate isomerase deficiency. In one instance, the level of monophosphoglycerate was determined in the elution profile following anion-exchange analysis of a blood filtrate from our patient. The value obtained (10.7 μmoles/100 ml. cells) is a normal value and represents the sum of 2-phosphoglycerate, 3-phosphoglycerate, and 1, 3-diphosphoglycerate within the erythrocyte. The sum of the various acid-soluble organic phosphates identified in blood filtrates from our patient accounted for 97.7 ± 2.7 per cent (average ± s.d.) of the total acid soluble phosphates determined by direct analysis of the blood filtrate. This finding indicates that there was no significant accumulation of any unusual phosphate ester within the erythrocytes. This evidence provides
strong support for the view that there is no deficiency of any of the enzymes in the glycolytic pathway of metabolism.

Recent studies have provided experimental verification of the view that adequate ATP levels are essential for red cell viability. There is a low ATP level due to impaired conversion of ADP to ATP in most cases of hemolytic anemia due to pyruvate-kinase deficiency.\textsuperscript{32,34-36} Seven of eight subjects with pyruvate-kinase deficiency studied by these workers showed reduced levels of erythrocyte ATP, and all showed ADP:ATP ratios which were above normal. Oski and Diamond\textsuperscript{34} also noted in their two subjects, an increased rate of fall in ATP level during in vitro incubation of red cells. In a recent study by Keitt,\textsuperscript{37} however, normal ATP levels were found in a patient with pyruvate-kinase deficiency with a high reticulocyte level. In this case, the ATP level was stable during in vitro incubation of erythrocytes for four hours at 37 C. However, ATP formation occurred primarily by oxidative phosphorylation in the mitochondria of the reticulocytes. When cyanide was added to prevent ATP formation by oxidative phosphorylation, the ATP level of the erythrocytes fell markedly. Recently, Lühr and Waller\textsuperscript{39} have described a family with a low level of ATP in erythrocytes. In this case, the decreased ATP level was apparently due to a low level of hexokinase. Many workers have shown that there is a correlation between ATP levels in stored red cells and the in vivo viability of these cells.\textsuperscript{38-40} If ATP levels are maintained during storage, viability of transfused cells remains high. If ATP level falls during storage, viability of transfused cells is poor. Since erythrocyte viability appears to be related to erythrocyte ATP levels in the different studies cited above, the low and unstable ATP noted in the patient described in this study is probably also related to the shortened life span of her erythrocytes. This low ATP level, however, is probably the result of a more fundamental cellular defect.

Low levels of ATP may be due to various factors. ATP is an essential energy repository for erythrocytes, as well as for all other cells of the body. It is required as an immediate source of energy for protein synthesis and other biosynthetic reactions, for ion transport against a concentration gradient, and for maintenance of the biconcave disc shape.\textsuperscript{41} The requirement for ATP for protein synthesis may vary markedly. Although there is probably no protein synthesis in mature erythrocytes, reticulocytes are active in synthesizing hemoglobin. Grimes et al.\textsuperscript{1} noted a rapid protein synthesis in erythrocytes of their patient with congenital Heinz-body anemia. Utilization of the energy of ATP in these various processes leads to formation of either AMP or ADP. In the red cell which is functioning properly, glycolysis provides the energy-rich phosphate compounds for the reformation of ATP by phosphate transfer to ADP from either 1, 3-diphosphoglycerate or from phosphoenolpyruvate. In addition, adenylate kinase is essential for the conversion of AMP to ADP. Consequently, it is clear that impairment in the activity of any of the glycolytic enzymes may produce a low level of ATP in erythrocytes. Low erythrocyte ATP levels would be an expected consequence of lowered levels of hexokinase or of pyruvate kinase when the activities of these enzymes are reduced sufficiently to become limiting.
Fig. 4.—Biosynthesis and catabolism of adenine nucleotides in human erythrocytes. Reactions 1 and 2 are biosynthetic reactions, and reactions 3, 4, and 5 are catabolic reactions. Adenine phosphoribosyl transferase catalyzes reaction 2 and AMP deaminase catalyzes reaction 3. Abbreviations, in addition to those given in the text are: PRPP, 5-phosphoribosyl 1-pyrophosphate; IMP, inosine monophosphate; and Rib, ribose.

Adenine nucleotide levels are also dependent upon a balance between the enzymes responsible for the biosynthesis of AMP and those responsible for AMP catabolism. The primary reactions of these sequences are summarized in Figure 4. In normal individuals in good health, the catabolic reactions are balanced by the anabolic reactions, and the level of total adenine nucleotides in erythrocytes stays essentially constant. Factors affecting AMP catabolism are poorly understood. AMP deaminase does not deaminate either ADP or ATP, so the portion of adenine nucleotides that is maintained as either the di- or triphosphate is not subject to this catabolic sequence. Any circumstance causing increases in the amount of AMP in erythrocytes leads to an increase in adenine nucleotide breakdown. Consequently, the level of substrate for AMP deaminase to act upon appears to be an important consideration. AMP deaminase is a rather unusual enzyme; it is activated by monovalent cations, but is inhibited by phosphate ion. At present, no good explanation can be presented correlating the in vitro properties of this enzyme with its function in initiating AMP catabolism in the intact erythrocyte.

In regard to the rate of biosynthesis of AMP, the limiting factor in vivo is probably the level of adenine in plasma, rather than the levels of the enzymes involved in the synthesis. On blood samples studied in this laboratory, the level of adenine in blood has been consistently below the lowest level which may be detected (ca. 1 μmole/100 ml. blood). It is possible that adenine levels in blood might be higher for a short period of time immediately after ingestion of foods containing nucleic acids. In vitro, adenine and inosine added to intact erythrocytes stimulate adenine nucleotide synthesis very dramatically. When the activity of adenine phosphoribosyl transferase (272 μmoles/100 ml. cells/hour), as noted in Table 2, is compared with the rate of adenine nucleotide breakdown in intact normal cells (1.3 μmoles/100 ml. cells/hour), as noted in Table 4, it is evident that the capacity of the enzyme primarily responsible for adenine nucleotide biosynthesis is more than adequate for the maintenance of erythrocyte adenine nucleotides when the substrate concentrations are not limiting.

In the patient described in this study, the low ATP level which was noted
appears to be due to an excessive rate of adenine nucleotide breakdown. The high glycolytic rate with no unusual accumulation of intermediates implies that ATP formation from ADP was proceeding rapidly. The following may be cited as evidence that there is no defect in the adenine nucleotide biosynthetic pathway: (1) the high level of adenine phosphoribosyl transferase and (2) the stimulus to adenine nucleotide synthesis noted when adenine was added to intact cells (Fig. 3).

The evidence presented in this study suggests that the defect in the patient's erythrocytes may involve an "overutilization" of ATP rather than "underproduction." The question in regard to the mechanism of utilization of ATP by the cell remains unanswered. Several hypotheses may be suggested:

1. There is an excessive requirement for ATP for active transport as a consequence of modification of the properties of the membrane; in this case, the increased membrane permeability might impose a larger energy requirement to maintain the increased load placed upon the cation pump.

2. There is an excessive requirement for ATP for biosynthetic processes (e.g., synthesis of proteins, membrane components, etc.).

3. ATP is lost (or not formed) as a consequence of excessive phosphatase activity, acting either on ATP or on one or more of the phosphate esters of the cell.

It is of interest to note that increased catabolism of adenine nucleotides is also a major metabolic manifestation of damage when erythrocytes are incubated in vitro with acetylphenylhydrazine. In this case, there is evidence that acetylphenylhydrazine damages membranes as well as cellular proteins.

SUMMARY

Studies have been carried out on erythrocytes of a young girl with a severe hemolytic anemia. Biochemically, these cells were characterized by a very high glycolytic rate, a low ATP level, an elevated ADP:ATP ratio, and a markedly increased rate of catabolism of adenine nucleotides to hypoxanthine during incubation in vitro.

Specific enzyme assays of the patient's cells revealed normal or elevated values for glucose-6-phosphate dehydrogenase, pyruvate kinase, glutathione reductase, enolase, adenosine triphosphatase, and adenine phosphoribosyl transferase.

In in vitro incubation studies, addition of adenine to the blood brought about an increase in the ATP level and in total adenine nucleotides. These data suggest that the low ATP level in the patient's blood was due to increased catabolism of adenine nucleotides, probably as a consequence of markedly increased utilization of ATP by the cell.

SUMMARIO IN INTERLINGUA

Esseva studiate le erythrocytos de un juvenile puera con un sever anemia hemolytic. Biochimicamente, le cellulas esseva caracterisate per un intensissime glycolyse, un basse nivello de ATP, un elevate proportion de ADP a ATP, e un marcatemente accelerate catabolismo de nucleotidas adeninic ad in hypoxanthine in incubation in vitro.

Le esssayage specific pro certe enzimas in le cellulas del patiente revelava normal o elevate valores pro dehydrogenase de glucosa-6-phosphato, kinase pyruvatic, reductase
HEMOLYTIC ANEMIA

31

glutathionic, enolase, triphosphatase de adenosina, e transferase adeno-phosphoribosylic.

In studios a incubation in vitro, le addition de adenina al sanguine effectuava un augmento in le nivello de ATP e de total nucleotidas de adenina. Iste datos suggestiona que le basse nivello de ATP in le sanguine del patiente esseva le effecto de un augmentate catabolismo de nucleotidas de adenina, probablemente in consequentia de un marcatemente augmentate utilisation de ATP per le cellula.

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Hemolytic Anemia Associated with Low Erythrocyte ATP

GORDON C. MILLS, WILLIAM C. LEVIN and JACK B. ALPERIN

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