Studies on the Hemolytic Nature of Protein-Deficiency Anemia in the Rat

By L. DELMONTE, A. ASCHKENASY AND A. EYQUEM

Rats subjected to total protein deprivation (PD) over a period of 7 to 10 weeks develop severe hematologic disturbances such as leukopenia and hypochromic microcytic anemia. The characteristics of this syndrome, observed as early as 1937 by Orten et al., have since been described in detail by both Orten and Aschkenasy and his colleagues. The anemia and leukopenia, due in part to an exhaustion of tissue reserves of amino acids essential to hemopoiesis and erythroid metabolism, is potentiated by a decrease in the level of certain hemopoietically active vitamins as well as by endocrine disturbances such as thyroid and gonad hypoactivity and relative adrenal hyperactivity.

Two possible mechanisms have been suggested for the anemia of experimental PD: (1) intramedullary inhibition of red cell maturation and/or release into the peripheral circulation; and (2) intravascular hemolysis of intra- or extracellular origin.

The hypothesis of an intramedullary block causing PD anemia is supported by the findings of (1) significant peripheral reticulocytosis during the first days of protein refeeding following PD and (2) an intramedullary maturation arrest at the erythroblast and reticulocyte stages. Such an arrest, noticed especially during the first two weeks of PD, appears much less pronounced after more prolonged protein deficiency when, even prior to protein refeeding, an increase in peripheral reticulocyte levels is observed. Erythrocyte Fe uptake shows the same pattern as peripheral reticulocyte numbers: a marked decrease during the first weeks, followed by a considerable increase during the late stages of PD. The paradoxic increase in bone marrow erythropoietic activity following protracted PD, paralleling a pronounced anemia, appears to be explained most readily by an increase in peripheral hemolysis—a hypothesis further substantiated by the observation of splenic siderosis in PD rats.

To test this hypothesis, we investigated the in vitro response to known hemolytic agents and the in vivo survival of PD erythrocytes. Our preliminary results indicated that a structural defect in these erythrocytes is not in itself sufficient to account for the severity of intravascular hemolysis.

Abnormal serum protein and lipid fraction concentrations were observed in the PD rat, a condition confirmed by more detailed studies of one of us (Aschkenasy) and Weimer et al. Consequently, we investigated the effects on the in vitro hemolytic behavior of PD erythrocytes of both whole serum and those serum fractions (albumin, cholesterol, lecithin) whose concentrations are most severely reduced in PD.

From the Hôpital de la Pitié and Institut Pasteur, Paris, France.
Submitted Aug. 9, 1963; accepted for publication Jan. 6, 1964.

Blood, Vol. 24, No. 1 (July), 1964
Materials and Methods

Male albino Wistar rats (180–190 Gm.), kept in individual cages at 23 C., were fed balanced synthetic diets containing 18 per cent devitaminized casein.* For studies on PD, the protein was replaced in toto by saccharose. Standard hematologic values (erythrocyte, hemoglobin and hematocrit levels) were determined from morning samples of caudal vein blood. Mechanically defibrinated or heparinized cardiac blood was used for mechanical fragility studies; heparinized or citrated blood for osmotic resistance studies; citrated aortic blood for all other hemolysis studies.

The hemoglobin concentration in the supernatant of hemolytic systems was determined with a Jobin and Yvon electrophotometer. Hemolysis was considered complete at 90 to 100 per cent and absent at 0 to 10 per cent lytic levels.

Neutral buffered† hypertonic saline (1.2 per cent NaCl) was used as the stock solution from which both isotonic and hypotonic salines were prepared as required. All erythrocyte samples were washed 3 times to remove traces of serum, and subsequently resuspended in buffered isotonic saline.

For studies of the antispherocytic factor (ASF), very dilute suspensions of washed erythrocytes were treated by Furchgott's method (1) adsorption of ASF on glass beads for 30 minutes, (2) filtration of ASF-free erythrocyte suspensions through several layers of gauze, and (3) washing and resuspension of the ASF-free erythrocytes in isotonic saline.

Sera from each nutritional group were pooled, lyophilized, and frozen until needed. In addition, individual samples of PD serum and control serum were analyzed by the biuret method for total protein, total lipid, cholesterol and lecithin concentration, and electrophoretically for serum albumin and globulin fraction values.

Hemolysin and serum fractions were stored at 4 C. for no longer than 1 week. Various concentrations of saponin (Merck, Darmstadt) and digitonin (Rhône-Poulenc, Paris) in 1.2 per cent saline as well as 2 mg. each of lecithin,† cholesterol (Rhône-Poulenc, Paris), and/or bovine serum albumin per ml. distilled water were prepared as stock suspensions.§

Just prior to use, 20 per cent dilutions of volumetrically reconstituted sera and 4 per cent suspensions of washed erythrocytes were made up with isotonic saline. Thus the final proportion of erythrocytes to pure serum in hemolytic systems containing equal parts of both suspensions was 1:5—comparable to the median hematocrit value of blood from PD rats.

Chemical hemolysins. The hemolytic systems contained, in order of delivery: (1) 1.6 ml. of hemolysin suspension, (2) 0.2 ml. of either isotonic saline or a 5 per cent serum dilution or a serum fraction suspension, and (3) 0.2 ml. of a 4 per cent erythrocyte suspension.

Five progressive dilutions of both saponin (50.0, 37.5, 25.0, 12.5 and 10.0 µg./ml.)

---

*Composition of diet (per 100 Gm. dry weight): 18.0 Gm. devitaminized casein (Byla), 61.3 Gm. saccharose, 10.0 Gm. white dextrine, 5.0 Gm. peanut oil, 5.7 Gm. mixed mineral salts (Orent-Keiles mixture #22), 25 mg. ascorbic acid, 1 mg. thiamine, 2 mg. riboflavin, 20 mg. nicotamide, 2 mg. pyridoxine, 3 mg. calcium pantothenate, 100 γ folic acid, 6 γ vitamin B₁₂, 60 mg. meso-inositol, 20 γ d-biotin, 1 mg. tocopherol, 1 mg. vitamin K, 500 I.U. vitamin A, 1,000 I.U. vitamin D, 500 mg. choline chloride. Final dilution: 100 Gm. dry weight diet per 50 ml. water.

†Ponder.**

1A mixture of oleic and stearic lecithin (Prolabo, Paris, France).

§Cholesterol and lecithin were first dissolved in small amounts of methyl alcohol to facilitate suspension.
HEMOLYTIC NATURE OF PROTEIN DEFICIENCY

and digitonin (5.00, 3.75, 2.50, 1.25 and 1.00 \( \mu \text{g.} / \text{ml.} \)) were used in these studies. At intervals up to 1\( \frac{1}{2} \) hours, hemolytic titers* were determined both microscopically, by hemocytometric counting of unlysed erythrocytes, and macroscopically, by electrophotometric hemoglobinometry.

The comparative 30-minute hemolytic titers of ASF-protected and ASF-free erythrocytes in 20 \( \mu \text{g.} / \text{ml.} \) of saponin and 2 \( \mu \text{g.} / \text{ml.} \) of digitonin were determined electrophotometrically in a similar system.

**Alkali sensitivity.** The hemolytic system contained: (1) 4.0 ml. of isotonic saline, (2) 0.5 ml. of either isotonic saline or a 5 per cent serum dilution, and (3) 0.5 ml. of a 4 per cent erythrocyte suspension. The system was gradually alkalized by adding one drop of 0.15 M NaOH every 5 minutes. The pH was measured immediately in an Adsul electropotentiometer; the per cent hemolysis in a hemocytometer. In a wet chamber, one drop of the suspension was allowed to settle for 5 minutes between slide and coverslip of (a) untreated and (b) siliconated glass, and studied for alkali-dependent changes in cell shape as described by Furchgott.37 All glassware for this procedure was chemically clean and stored in neutral distilled water until used.

**Osmotic resistance.** A series of hemolysis tubes were set up, containing: (1) 2 ml. of neutral saline (progressive dilutions: 0.90, 0.85, . . . 0.05, 0.00 Gm. per cent NaCl),† (2) 0.25 ml. of either isotonic saline or a 5 per cent serum dilution, and (3) 0.25 ml. of a 4 per cent erythrocyte suspension. After 30 minutes, the system was centrifuged (1000 rpm x 5 min.) and hemolysis measured by electrophotometric determination of the hemoglobin concentration in the supernatant.

**Mechanical fragility (M F).** A 50-ml. Erlenmeyer flask containing four 3-mm. glass beads and 0.5 ml. of a 35 per cent erythrocyte suspension in either hypertonic saline or 13 per cent PD or control serum dilution was rotated horizontally at 90 rpm x 1 hour at 23 C.‡ Both heparinized blood, collected in untreated or siliconated glassware, and mechanically defibrinated blood were studied. For each sample, the following hemolytic systems were prepared:

**A**—0.1 ml. unrotated RBC suspension + 1 ml. distilled water

**B**—0.1 ml. rotated RBC suspension + 1 ml. of 1.2 per cent NaCl solution

**C**—0.1 ml. unrotated RBC suspension + 1 ml. of 1.2 per cent NaCl.

Following centrifugation (1000 rpm x 5 min.), the concentration of hemoglobin in the supernatants A, B and C was determined electrophotometrically, and the mechanical fragility (MF) calculated according to Shen’s formula:

\[
MF = \frac{(B - C) \times 100}{(A - C)}
\]

**Erythrocyte survival.** Samples of heparinized blood (0.6 ml.) were taken from 10 PD and 10 control rats. Pooled samples (1.2 ml.) from two rats were incubated for 1 hour at 23 C. with 1 ml. of an isotonic saline solution of Na\(_2\)Cr\(_{51}\)O\(_4\) having a specific activity of 216 \( \mu \text{g.} / \text{ml.} \). The suspension was gently agitated with a glass rod every 15 minutes. No increase in erythrocyte labeling could be obtained by doubling the Cr\(_{51}\) concentration and the incubation time. After triple-washing to remove traces of unadsorbed Cr\(_{51}\), the

---

*Read as 8.0 to 40.0 \( \mu \text{g.} / \text{ml.} \) of saponin and 0.8 to 4.0 \( \mu \text{g.} / \text{ml.} \) of digitonin, since the final ratio of hemolysin suspension to the total volume of the hemolytic system is 4:5.

†Read as 0.72, 0.60, . . . 0.04, 0.00 Gm. per cent NaCl) since the final ratio of hypotonic saline to the total volume of the hemolytic system is 4:5.

‡Apparatus from six flasks, having a 15-cm. radius from the axis of rotation to the center of the flask bottom, constructed in the laboratory of one of us (Dr. A. Eyquem, Institut Pasteur, Paris, France), according to the specifications of Shen et al.72 as modified by Young et al.81 and Gardner et al.30
labeled cells were reconstituted to their original hematocrit concentration in isotonic saline. One ml. of the labeled erythrocyte suspension was injected into the saphenic vein of intact receiver rats (140-180 Gm.) fed balanced diets. At specific intervals thereafter (1 hour, and 2, 4, 7 and 14 days), the activity of caudal vein blood samples was measured in a Tracerlab P-20 scintillation counter. Erythrocyte survival was calculated from the formula:

\[
\text{% RBC survival} = \frac{\text{radioactivity of 0.2 ml sample on day 1} \times 100}{\text{radioactivity of 0.2 ml sample at 1 hour}}
\]

**RESULTS**

Rats maintained on the PD diet for 50 days showed a moderate but already significant anemia: 6.33 ± 0.13 million RBC/cu. mm.* and 9.5 ± 0.5 Gm. Hb/100 ml., instead of normal values of approximately 8 million RBC/cu. mm. and 15 Gm. Hb/100 ml., respectively. Body weight decreased from 180-190 Gm. to 100-120 Gm.

*Mean ± standard error.

**Hemolytic Behavior of Rat Erythrocytes In Vitro**

**Chemical hemolysis.** The erythrocytes of PD rats showed a reduced resistance in vitro to hemolysis by both saponin and digitonin. The hemolysin titers necessary for complete lysis within a given period of time were lower for PD than for control erythrocytes. Lytic titers of saponin leveled off at 20 ± 0 µg./ml. and 25 ± 2 µg./ml. for PD and control erythrocytes, respectively, after 90 minutes (fig. 1A). Lytic titers of digitonin leveled off at 2.1 ± 0.2 µg./ml. after 90 minutes for PD erythrocytes, and at 3.4 ± 0.4 µg./ml. after 30 minutes for control erythrocytes (fig. 1B).

For erythrocyte-lysin systems containing a fixed concentration of saponin (20 µg./ml.) or digitonin (2 µg./ml.) time-hemolysis curves were biphasic (fig. 2). An initial 15-minute period of rapid hemolysis was followed by a period of gradual leveling off. PD erythrocytes manifested an accelerated rate of lysis; their hemolysis was complete within 30 minutes when only 35 to 40 per cent of the control cells had lysed.

The removal of ASF from the cell surface nearly doubled the susceptibility of even normal erythrocytes to saponin and digitonin lysis. However, the ASF-free normal erythrocytes' resistance to chemical lysis was still significantly greater than that of ASF-coated PD erythrocytes (table 1).

**Alkali sensitivity.** Figure 3 indicates that PD erythrocytes are abnormally sensitive to alkalinity. Both the onset and end point of hemolysis were lower for PD (pH 8.8 and pH 10.8, respectively) than for control erythrocytes (pH 10.2 and pH 11.0, respectively). Comparable erythrocytes shape changes—ranging from the biconcave disc, through the crenated forms, to the spherocyte—occurred at less alkaline pH values in PD than in control erythrocytes (table 2).

Removal of ASF by unsiliconated glass surfaces, although not significantly affecting these alkali-dependent shape changes, did appear to increase the amount of individual variation occurring within the PD group.
Fig. 1.—Hemolytic titer of saponin and digitonin as a function of time of contact with erythrocytes of rats on control and protein-deficient (PD) diets (electrophotometric method) (M. ± S.E.)

Osmotic resistance. The osmotic resistance of PD erythrocytes was significantly greater than that of control cells (fig. 4), and individual variations were greater in the former than in the latter experimental group. It is interesting to note that, whereas the osmotic resistance of normal erythrocytes remained unaffected by the anticoagulant used, the osmotic resistance of PD erythrocytes was greater in heparinized than in citrated blood (fig. 4).

Mechanical fragility. For both the control and the PD group, the MF was much more pronounced in erythrocytes from defibrinated than from heparinized blood. Regardless of the method of inhibiting clot formation, the MF was about 1½ times as great for PD as for control erythrocytes (table 3).

The MF of normal erythrocytes was not significantly affected by ASF-adsorption unto unsiliconated glass surfaces during the process of mechanical traumatization.

Effect of Serum and Certain Serum Fractions on the Hemolytic Behavior of Rat Erythrocytes In Vitro

Sera of protein-depleted (60 days) rats contained a total protein concen-
Fig. 2.—Per cent hemolysis as a function of time of contact of saponin (20 μg./ml.) and digitonin (2 μg./ml.) with erythrocytes of rats on balanced and protein-deficient (PD) diets (hemocytometric method) (M. ± S.E.).

Chemical hemolysis. Normal serum was found to protect control but not PD erythrocytes against the lytic effects of saponin (24 μg./ml.). Digitonin hemolysis (2.4 μg./ml.) of control and PD cells was inhibited by control as well as PD serum. Against the given concentration of lysin, the protective effect of normal serum was greater than that of PD serum (figs. 5A and 5B). The antihemolytic protection of serum was greater for control than for PD erythrocytes.

Of the three serum fractions studied (figs. 5A and 5B), lecithin was the most potent protective agent against chemical hemolysis, comparable or even superior to that of normal serum. As in the case of serum, this antihemolytic effect was more pronounced on normal than on PD erythrocytes.

For control erythrocytes, cholesterol proved less effective than PD serum against saponin hemolysis (fig. 5A), and as effective as PD serum against digitonin hemolysis (fig. 5B). For PD erythrocytes, cholesterol was slightly more effective than either serum against saponin hemolysis and potentiated the antihemolytic effect of lecithin (fig. 5A); but cholesterol was slightly less effective than normal or PD serum against digitonin hemolysis (fig. 5B).

Albumin failed to protect the normal erythrocytes against saponin and normal as well as PD cells against digitonin hemolysis (figs. 5A and 5B). Moreover, albumin reduced the efficacy of cholesterol against PD erythrocyte lysis by digitonin.
HEMOLYTIC NATURE OF PROTEIN DEFICIENCY

Table 1.—Effect of Antisphering Factor (ASF) on Chemical Hemolysis of Erythrocytes of Rats on Balanced and Protein-Deficient (PD) Diets

<table>
<thead>
<tr>
<th>Lysin Suspension</th>
<th>ASF</th>
<th>Hemolysis (%) at 30 Minutes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Control RBC</td>
</tr>
<tr>
<td>Saponin (20 µg./ml.)</td>
<td>+</td>
<td>32.9 ± 3.0</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>51.9 ± 4.4</td>
</tr>
<tr>
<td>Digitonin (2 µg./ml.)</td>
<td>+</td>
<td>38.1 ± 4.6</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>74.5 ± 6.1</td>
</tr>
</tbody>
</table>

It is interesting to note that PD erythrocytes, refractory to the antihemolytic effect of both control and PD serum against saponin, still retain their capacity to respond to the protective action of physiologic concentrations of lecithin and cholesterol.

Alkali sensitivity. Serum increased but did not normalize the alkali-resistance of PD erythrocytes (fig. 6). Control serum was more effective than PD serum. Control but not PD serum also increased the alkali-resistance of control erythrocytes.

Osmotic resistance. Serum failed to alter significantly the osmotic resistance of either control or PD erythrocytes.

Mechanical fragility. Control serum was found to reduce MF. This effect was similar with both PD and control erythrocytes. PD serum, on the other hand, failed to affect the MF of either type of erythrocyte (table 5).

In Vivo Survival of Cr51-labeled Rat Erythrocytes

Figure 7 shows that PD erythrocytes were destroyed more rapidly than control cells. The destruction of PD erythrocytes preceded that of the control cells by about 3 to 7 days: Approximately one-half of the Cr51 activity disappeared within 6 days from PD and within 9 days from control erythrocytes; barely one-quarter of the activity still was measurable in PD cells at the end of 14 days, whereas over one-quarter of the activity remained in control cells at the end of 21 days.

Two recipient rats—one from each experimental group—were excluded from the analysis because the rate of Cr51 disappearance indicated incompatibility between donor and recipient blood.

DISCUSSION

Our results show an abnormal hemolytic behavior of PD erythrocytes in vitro, thus tending to support the concept of a structural defect leading to hemolysis of the cell in vivo.

We limited our studies to two chemical lysins (saponin and digitonin) whose kinetics are well documented. Ponder and Ruhenstroth-Bauer showed that when the temperature, pH, and cell concentration remain constant, the hemolytic lysin titer depends on (1) the degree of red cell surface saturation with lysin molecules, and (2) the time of exposure to the lysin. Compared to the controls, the PD erythrocytes appeared to hemo-
Fig. 3.—Alkali resistance of erythrocytes of rats on balanced and protein-deficient (PD) diets (M. ± S.E.).

lyse at lower lysin (saponin and digitonin) concentrations over a given period of time and in less time for a given lysin concentration. This phenomenon has been confirmed by Aschkenasy and Blanpin who, however, reported higher hemolytic titers. The latter is probably due to differences in the rat strains used as well as to the more profound depletion of our rats which presented lower serum protein and lipid concentrations. Ponder and Rhoads reported a similar abnormal erythrocyte behavior towards saponin, sodium taurocholate and sodium glycholate developing in certain patients with pernicious anemia.

While ASF interfered with the hemolytic efficacy of the saponins, its removal from the surface of both PD and control erythrocytes reduced but did not suppress the difference in lysin susceptibility of the two experimental groups. It is not known whether ASF interacts directly with the lysin or, in coating the cell membrane, reduces the concentration of free cholesterol molecules available for binding the lysin molecules.

Erythrocyte shape changes in media for increasing alkalinity reflect a progressive hemolytic process. As the alkalinity increases, the molecular ultrastructure of the erythrocyte membrane loses its normal orientation, so that the cell can no longer maintain its biconcave disc architecture; the erythrocyte becomes crenated, a phenomenon which Teitel-Bernard attributed to hemoglobin crystallization. Gradually the osmotic equilibrium between the colloid phase within the cell and the liquid phase of the suspension medium is upset at the membrane interphase. The surface tension and the erythrocyte volume increase without a corresponding increase occurring in the cell surface area; the erythrocyte becomes spherocytic. Finally, an irreversible stage, the critical volume, is reached; hemoglobin diffuses
HEMOLYTIC NATURE OF PROTEIN DEFICIENCY

Table 2.—Alkali Dependence of Shape of Erythrocytes of Rats on Balanced and Protein-deficient (PD) Diets, as Modified by Antisphering Factor (ASF)

(D = disc, CD = crenated disc, CS = crenated sphere, S = sphere)

<table>
<thead>
<tr>
<th>pH</th>
<th>D</th>
<th>D-CD</th>
<th>CD</th>
<th>CD-CS</th>
<th>CS</th>
<th>CS-S</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>D</td>
<td>D-CD</td>
<td>CD</td>
<td>CD-CS</td>
<td>CS</td>
<td>CS-S</td>
</tr>
<tr>
<td>7.0</td>
<td>6</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7.2</td>
<td>6</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7.4</td>
<td>6</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7.6</td>
<td>6</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7.8</td>
<td>6</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8.0</td>
<td>6</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8.2</td>
<td>6</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8.4</td>
<td>6</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8.6</td>
<td>6</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8.8</td>
<td>6</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9.0</td>
<td>6</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9.2</td>
<td>6</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9.4</td>
<td>6</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9.6</td>
<td>6</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9.8</td>
<td>5</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10.0</td>
<td>4</td>
<td>2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10.2</td>
<td>4</td>
<td>2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10.4</td>
<td>2</td>
<td>3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10.6</td>
<td>1</td>
<td>4</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10.8</td>
<td>1</td>
<td>2</td>
<td>2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11.0</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11.2</td>
<td>6</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

A. ASF + (siliconated slide and coverslip)

<table>
<thead>
<tr>
<th>pH</th>
<th>D</th>
<th>D-CD</th>
<th>CD</th>
<th>CD-CS</th>
<th>CS</th>
<th>CS-S</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.0</td>
<td>6</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7.2</td>
<td>6</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7.4</td>
<td>6</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7.6</td>
<td>6</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7.8</td>
<td>6</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8.0</td>
<td>6</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8.2</td>
<td>6</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8.4</td>
<td>6</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8.6</td>
<td>6</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8.8</td>
<td>6</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9.0</td>
<td>6</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9.2</td>
<td>6</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9.4</td>
<td>6</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9.6</td>
<td>6</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9.8</td>
<td>5</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10.0</td>
<td>4</td>
<td>2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10.2</td>
<td>4</td>
<td>2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10.4</td>
<td>2</td>
<td>3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10.6</td>
<td>1</td>
<td>4</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10.8</td>
<td>1</td>
<td>2</td>
<td>2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11.0</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11.2</td>
<td>6</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

B. ASF - (unsiliconated slide and coverslip)
across the distorted cell membrane and only a stromatic ghost cell remains.

Erythrocyte shape changes found in the rat more or less parallel those reported by Furchgott in human erythrocytes at corresponding pH values. Upon removal of FAS, however, the rat erythrocytes—unlike those of man—show only slightly increased alkali sensitivity. Neutral and siliconated glassware, used in our studies, sufficed to avoid the development of abrupt shape changes such as many investigators have reported.

The exaggerated alkali sensitivity of the PD erythrocyte must be attributed to a defect in the cell membrane. For, whereas these cells' stroma presents a slightly increased concentration in proteins and phospholipids, their hemoglobin manifests a normal electrophoretic pattern and alkali resistance. And ASF removal has a comparable effect on the alkali sensitivity of PD and control erythrocytes. The high degree of individual variation in the alkali sensitivity appears to reflect the variable degree of hemolytic anemia found in the rats after 50 days of PD.

Jacobs and Parpart reported the osmotic resistance is much affected by changes in alkalinity. Our observations tend to confirm this finding. We therefore controlled this factor of variability by adding a neutral buffer, at the risk of introducing a small measure of ionic impurities (K+, Na+, and PO₄⁻) into the hypotonic solutions.

In contrast to its exaggerated sensitivity to chemical lysins, alkali, and mechanical trauma, the PD erythrocyte has an increased osmotic resistance. The available evidence concerning the relationship between erythrocyte age and osmotic resistance is too contradictory for us to hazard an opinion relating the reticulocytosis of PD blood to its high osmotic resistance. It is quite likely that the latter reflects the changes in proteins and phospholipids found by Aschkenasy et al. in the PD erythrocyte membrane.
HEMOLYTIC NATURE OF PROTEIN DEFICIENCY

Table 3.—Mechanical Fragility, in Presence and Absence of Antisphering Factor (ASF), of Erythrocytes of Rats on Balanced and Protein-deficient (PD) Diets, Following Traumatization at 90 rpm for 60 Minutes

( M. ± S.E.)

<table>
<thead>
<tr>
<th>Anticoagulant Method</th>
<th>ASF</th>
<th>No. of Rats</th>
<th>Control RBC</th>
<th>PD RBC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mechanical defibrinization</td>
<td></td>
<td></td>
<td>31.1 ± 2.7</td>
<td>52.3 ± 3.3</td>
</tr>
<tr>
<td>Heparinization</td>
<td></td>
<td></td>
<td>17.7 ± 3.0</td>
<td>27.6 ± 3.1</td>
</tr>
</tbody>
</table>

*ASF adsorption from erythrocytes prevented by siliconation of glassware.

Normal rat erythrocytes can be rendered hyperresistant to hypotonic saline by artificial conversion of their hemoglobin to a paracrystallin state at low temperatures. However, the normal hemoglobin picture found in the PD rat erythrocyte tends to preclude ascribing the cells' osmotic hyperresistance to an abnormal crystallization phenomenon of its hemoglobin.

Furthermore, the osmotic resistance of the normal rat erythrocytes is not affected by the type of anticoagulant used, whereas that of the PD erythrocyte is.

These findings appear to rule out all but a structural defect of the cell membrane as the cause of the osmotic hyperresistance of the PD erythrocyte.

Because of the diversity of apparatus for mechanical traumatization which are used in different laboratories, we felt that our data, while obtained with an apparatus calibrated sufficiently according to established standards to permit reliable comparison of MF values of control and PD erythrocytes, cannot be compared quantitatively with that of other investigators. Nor can the in vitro trauma be equated to that which the erythrocytes undergo in vivo. Nevertheless, in vitro measurements of MF do furnish valuable information as to the degree to which the altered membrane of the PD erythrocyte still retains its ability to withstand mechanical trauma.

Shen et al. observed a direct relationship between the erythrocytes' MF, permeability and membrane architecture. Increased MF has been observed to occur in various human hemolytic anemias including the spherocytic and sickle cell forms. Stewart et al. demonstrated an inverse relationship between the age and the MF of dog erythrocytes labeled with radioactive iron. Our studies revealed no difference in the degree of protection against mechanical trauma which ASF confers on the PD and control erythrocytes. Thus the implication of a structural defect in the exaggerated mechanical fragility of the PD erythrocyte appears to be quite clear.

Serum contains substances capable both of accelerating and inhibiting hemolytic processes. Its antihemolytic potency is dependent on biochemical interaction of the serum constituents with each other, with the hemolytic agent, and with the components of the erythrocyte cell membrane.

Hence our findings of abnormalities in the constitution of PD serum, which
agree with an earlier report\textsuperscript{14} of a serum albumin decrease and have since been confirmed by the more complete studies of Aschkenasy and Blanpin\textsuperscript{15} and Weimer et al.\textsuperscript{8} made it seem likely that not only defective erythrocyte structure but also defective serum constitution is involved in the mechanism of PD anemia. Our animals appear to have been more severely depleted than those of Aschkenasy and Blanpin,\textsuperscript{15} as evidenced both by their higher degree of serum deficiencies and by their greater lysin sensitivity. The serum analysis led us to compare the relative antihemolytic efficacy not only of control and PD serum but also of the three most severely affected serum fractions (albumin, cholesterol and lecithin).

Ponder\textsuperscript{58,59} reported divergent antihemolytic potency of human serum regarding hemolysis by saponin and related substances, and suggested that...
HEMOLYTIC NATURE OF PROTEIN DEFICIENCY

Fig. 6.—Effect of control and protein deficient (PD) whole serum on alkali resistance of erythrocytes of rats on balanced and PD diets (M. ± S.E.).

This potency—apparently related to cholesterol, lecithin and protein concentration—might be reduced in nutritional deficiency states. This was confirmed by our data as well as those of Aschkenasy and Blanpin\textsuperscript{15} and Weimer et al.\textsuperscript{80} While both sera confer some protection against chemical hemolysis, the potency of PD serum is considerably lower. It is evident, however, that the antihemolytic potency of either serum is definitely limited by the lysin-sensitivity of the PD erythrocytes themselves. Unlike human erythrocytes, rat erythrocytes receive greater antisaponin protection from lecithin than from cholesterol. This merely confirms Ponder's\textsuperscript{59} findings that cholesterol protects human erythrocytes better than rat erythrocytes against saponin hemolysis. In the rat, lecithin appears to be a prime protective factor against saponin and digitonin hemolysis. The potency of lecithin is only slightly augmented by the addition of cholesterol which is per se ineffective against saponin and only moderately effective against digitonin at the lysin titers investigated.

Like human serum albumin,\textsuperscript{59} bovine serum albumin does not inhibit chemical hemolysis, and cannot be identified with the rat ASF, the factor which Furchgott\textsuperscript{87} considered to be Hewitt's\textsuperscript{46} crystalbumin and whose antisaponin and anti-digitonin potency we have demonstrated. Nor can bovine serum albumin be identified without further study with albumin present in rat serum.

Lecithin is fully and cholesterol only partially effective against digitonin lysis of PD erythrocytes in the presence of albumin.
Table 5.—Inhibitory Effect of Serum on Mechanical Fragility of Erythrocytes of Rats on Balanced and Protein-deficient (PD) Diets, Following Traumatization at 90 rpm for 60 Minutes

(M. ± S.E.)

<table>
<thead>
<tr>
<th>“Inhibitor”</th>
<th>Hemolysis (%) Control RBC</th>
<th>PD RBC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isotonic saline</td>
<td>17.7 ± 3.0</td>
<td>27.6 ± 3.1</td>
</tr>
<tr>
<td>Control serum</td>
<td>10.2 ± 1.3</td>
<td>15.2 ± 2.6</td>
</tr>
<tr>
<td>PD serum</td>
<td>15.1 ± 5.3</td>
<td>19.7 ± 4.3</td>
</tr>
</tbody>
</table>

From the above, it appears that the reduced antihemolytic potency of PD serum may be due primarily to its greatly reduced lecithin, and secondarily to its reduced cholesterol content.

Normal serum partially protects both normal and PD erythrocytes against alkali hemolysis. However, the alkali sensitivity of PD erythrocytes in control serum still remains greater than that of normal rat erythrocytes in isotonic saline. This places in perspective the relative importance of erythrocyte and serum anomalies in the exaggerated alkali hemolysis of PD blood.

Human erythrocytes appear to be protected against alkali hemolysis by crystalbumin—presumably Furchgott's ASF. However, ASF fails to protect the rat erythrocytes. Consequently, it must be assumed that other serum factors are involved in the protection of rat erythrocytes against alkali hemolysis.

Osmotic fragility does not appear to be affected by the presence or absence of serum. A similar finding has been reported in the rabbit but not in man. Thus the osmotic hyperresistance of PD erythrocytes can only be due to intrinsic cellular factors controlling membrane permeability.

The mechanism of inhibition of the abnormal MF of PD erythrocytes, partial with PD and complete with normal serum, is not known. Since osmotic resistance is not involved, and since other factors of variability known to affect the MF remain constant, only serum factors—more specifically, only serum factors reduced in concentration in PD serum—can be involved directly. For in control serum, the MF of PD erythrocytes returns to normal, indicating that the cells, although themselves abnormally sensitive to mechanical trauma, retain the ability to respond normally to the protective action of serum factors.

Instead of measuring the rat erythrocyte life span, as numerous investigators have already done with various radioisotopes, we limited ourselves to comparing the rate of in vivo destruction of transfused heterogenous Cr-labeled erythrocyte populations from control and PD donors. Mechanical trauma was reduced to a minimum by using gentle agitation with a glass rod during in vitro labeling, rather than the mechanical shaker advocated in the literature. Elution of Cr from the red cells—6 to 7 per cent per day, according to Berlin et al.—as well as loss of Cr activity in the 0.2 ml. blood samples taken periodically, was considered to be approximately equal for both experimental groups.
Our findings, while divergent from those of Belcher, agree with those of both Donohue et al. and Hall et al. who reported a 5- to 10-day Cr half-time for in vitro-labeled transfused normal rat erythrocytes.

Normal red cell survival has been shown to depend on cell age and on various factors of random destruction in vivo. Reticulocytes, whose number is increased in the late stage of PD, normally tend to survive longer than adult cells. However, PD red cells were destroyed more rapidly than control cells. Serum has no detrimental effect upon red cells in vitro, and is thus not likely to be involved in the increased in vivo destruction of red cells formed before or after the beginning of PD. In fact, as our in vitro data indicate, the normal serum of the recipient's blood would tend to protect rather than injure the circulating transfused PD erythrocytes. Thus one may reasonably draw the conclusion that the red cells formed after the beginning of protein inanition had a structural defect which rendered them abnormally sensitive to in vivo hemolytic agents. Most of the abnormal cells appear to be destroyed during the 1st week following transfusion when the older erythrocytes in both experimental series are also eliminated. This can be visualized by the steeper slope of phase 1 of the PD erythrocyte survival curve as compared to that of the control series; phase 2 appears to represent mainly random destruction of normal cell populations in both experimental groups.

Hamilton's findings that pernicious anemia is accompanied by both a partial maturation arrest in the bone marrow and increased hemolysis suggest an analogy of this dyscrasia with protein deprivation anemia. However, in the rat, neither folic acid nor vitamin B₁₂ deprivation are involved in the
etiology of the latter anemia, as is evident by its development despite the presence of adequate levels of both vitamins in the protein-free diet. Moreover, the characteristic hypochromia and microcytosis of protein deficiency anemia are in direct opposition to the characteristic features (hyperchromia and megalocytosis) of pernicious anemia.

In the light of recent knowledge concerning the role of enzymatic deficiencies in the pathogenesis of certain hemolytic anemias, an investigation into changes in the enzymatic and metabolic constitution of erythrocytes of protein-deprived animals might prove to be of interest.

**SUMMARY**

The hemolytic nature of experimental protein deprivation (PD) anemia has been established (1) by in vitro evidence of (a) the abnormal hemolytic behavior of the PD erythrocytes and an impairment of their reactivity to the protective effect of normal serum and certain of its fractions, and (b) the defective antihemolytic potency of PD serum towards both PD and normal erythrocytes; and (2) by in vivo evidence of the PD erythrocytes' decreased survival potential.

The hypersensitivity of the PD erythrocytes to chemical and mechanical trauma is interesting, in view of the fact that the cell population is heterogeneous and during the late stage actually includes a slightly elevated percentage of young forms which, normally, should be less sensitive to such trauma. Hence the findings of decreased chemical, alkali and mechanical resistance and increased osmotic resistance in vitro, decreased in vivo survival potential, and impaired responsiveness to in vitro protection by normal serum, lecithin and cholesterol against hemolytic factors, all point to a cellular origin, namely a structural defect, as the cause of the hemolytic nature of experimental PD anemia.

It is probable that the defective composition of PD serum which is less effective than normal serum in protecting normal erythrocytes against chemical, alkali and mechanical trauma in vitro, also contributes to the hemolytic nature of experimental PD anemia in the rat.

**SUMMARIO IN INTERLINGUA**

Le natura hemolytic de anemia experimental per deprivation de proteina (DP) eseva establite per studios (1) in vitro e (2) in vivo.
1. Le studios in vitro produceva evidentia (a) de anormalitates de comportamento hemolytic del erythrocytos DP e de un declino de lor reactivitate al effortio protectori de sero normal e de certes de su fractiones e (b) de defectos in le potentia antihemolytic de sero DP pro erythrocytos tanto DP como etiam normal.

2. Le studios in vivo produceva evidentia de un reduction in le potential de superviventia del erythrocytos DP.

Le hypersensibilitate del erythrocytos DP pro trauma chimic e mechanic es interessant in visa del facto que le population de cellulas es heterogenee e, durante le stadios tardive, include de facto un levemente aug-
HEMOLYTIC NATURE OF PROTEIN DEFICIENCY

mentate procentage de juvene formas que, normalmente, deberea esser minus sensibile pro tal trauma. Per consequent le constatation de reducite resistentia chimic, alcalin, e mechanic e de augmentate resistentia osmotic in vitro, de un reduceite potential de superviventia in vivo, e de un defective responsivitate in vitro al protection per sero normal, lecithina, e cholesterol contra factores hemolytic indica omnes un origine cellular, i.e. un defeceto structural, como causa del natura hemolytic de anemia experimental a DP.

Il es probable que le composition defective de sero DP que es minus effi-
cace que sero normal in proteger erythrocytos normal contra trauma chimic, alcalin, e mechanic in vitro etiam contribue al natura hemolytic de anemia experimental a DP in le ratto.

REFERENCES

15. —, and Blanpin, O.: Influence de la teneur du régime en graisses sur l’anémie et l’hémolyse ainsi que sur les taux des protéines, du cholestérol et des phospholipides sériques chez des...
38. —, and Ponder, E.: Disc-sphere transformations in mammalian red cells. II. The nature of the anti-sphering factor. J. Exper. Biol. 17:117-127,
HEMOLYTIC NATURE OF PROTEIN DEFICIENCY

1940.


56. —: The mechanism of the inhibition of hemolysis. J. Gen. Physiol. 27:1-14, 1943.

57. —: The osmotic behavior of crenated red cells. J. Gen. Physiol. 27:273-385, 1944.


60. —: The paracrystalline state of the rat red cell. J. Gen. Physiol. 29:89-102, 1946.


64. —, and Neurath, H.: On the spherical form of the mammalian erythrocyte. IV. Disc-sphere transformations be-


A. Aschkenasy, M.D., Sc.D., Research Director, Centre National de la Recherche Scientifique, Hôpital de la Pitié, Paris.

A. Eyquem, M.D., Head of the Department of Hematology, Institut Pasteur, Paris.

L. Delmonte, Sc.D., Department of Anatomy, Baylor University School of Medicine, Houston, Texas.
Studies on the Hemolytic Nature of Protein-Deficiency Anemia in the Rat

L. DELMONTE, A. ASCHKENASY and A. EYQUEM