Selective Reticulocyte Destruction in Erythrocyte Pyruvate Kinase Deficiency

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A B S T R A C T   Radioisotope studies of bilirubin turn-over, ferrokinetics, and red cell survival (^{51}Cr) in a patient with erythrocyte PK deficiency have provided evidence for prompt reticulocyte sequestration and destruction by the reticuloendothelial system. More mature erythrocytes appeared to survive well despite their deficiency of PK. PK-deficient reticulocytes, dependent upon oxidative phosphorylation for ATP production, are exquisitely sensitive to cyanide- or nitrogen-induced mitochondrial inhibition. If oxidative phosphorylation is unavailable, ATP levels decline rapidly, producing alterations in the cell membrane which allow massive losses of potassium and water. The result is a shrunken, spiculated, viscous cell whose rheologic properties would favor its sequestration by the reticuloendothelial system. Those reticulocytes with particularly low levels of PK exhibit very low glycolytic rates and thus are uniquely reliant upon oxidative phosphorylation. Other reticulocytes, better endowed with PK activity, can meet the increased ATP requirements of young erythrocytes. Upon reaching maturity, such cells have diminished ATP needs and can, therefore, survive despite their enzyme deficiency.

INTRODUCTION

A specific deficiency of erythrocyte pyruvate kinase (PK) has been demonstrated in many individuals with congenital nonspherocytic hemolytic anemia (1). It has been postulated that aging of PK-deficient erythrocytes, which is associated with further attenuation of initially low levels of PK, rapidly renders such cells incapable of adequate glycolysis (1). Unable to maintain ATP levels, the deficient erythrocyte would soon be destroyed. PK-deficient reticulocytes, by contrast, possess mitochondria and the enzymes of the Krebs cycle. It has been demonstrated that oxidative phosphorylation enables the PK-deficient reticulocyte to maintain ATP despite inadequate glycolysis (2). With maturation, mitochondria are lost, and the cell then becomes doomed by its enzyme defect.

Several observations suggest, however, that the PK-deficient reticulocyte may be more, rather than less, liable to destruction. Studies of young and old PK-deficient erythrocytes, separated by centrifugation and differentially labeled with either ^{51}Cr or glycine-^{14}C, indicate that young PK-deficient erythrocytes may survive less well in vivo than more mature cells (3). Splenectomy, often associated with improved erythrocyte survival, is also frequently attended by persistently increased reticulocyte numbers. Indeed the reticulocyte count may on occasion exceed 90% (4, 5). The only reported examination of the spleen for reticulocytes revealed a marked increase in absolute numbers of reticulocytes in the splenic pulp of the single individual studied (6). These observations indicate that the PK-deficient reticulocyte may be selectively sequestered in the spleen where they undergo irreversible damage and ultimately premature destruction.

This paper provides further evidence for enhanced reticulocyte destruction in PK deficiency. In one patient direct evidence for the presence of a rapidly destroyed pool of newly formed erythrocytes was obtained by studies of erythrocyte turnover utilizing ^{59}Fe, bilirubin-

³H, and ⁵¹Cr-labeled erythrocytes. Cells that survived the reticulocyte stage then appeared to have a normal life span despite their deficiency of PK. The undue vulnerability of PK-deficient reticulocytes sequestered within the spleen was investigated in vitro by incubation of the blood of five splenectomized PK-deficient patients under conditions thought to stimulate certain features of the environment of the spleen. Physiological levels of hypoxia were found capable of severely compromising the metabolism of the PK-deficient reticulocyte, resulting in a cell with abnormal rheologic properties.

We have concluded from these studies that the limitation imposed by diminished erythrocyte PK activity depends upon the metabolic requirements of the cells in which the deficiency is present. Young red cells, whose metabolic requirements are greater than those of mature cells, appear to be considerably more sensitive to PK deficiency than are their more mature counterparts. Thus the absolute level of PK activity need not necessarily influence the life span of the erythrocyte.

METHODS

Subjects. Blood was collected in preservative free heparin (0.1 mg/ml of blood) for in vitro studies from five PK-deficient patients. All had undergone splenectomy at least 6 months before study and had received no transfusions postsplenectomy. Isotope measurements of mean red cell life span were carried out on a sixth PK-deficient patient whose anemia was mild, and who consequently had not undergone splenectomy. Representative hematologic data are shown in Table I. Control blood was obtained from both pre- and postsplenectomy patients with reticulocytosis. A variety of hematologic disorders were represented: nutritional anemia responding to therapy, myeloid metaplasia, osteopetrosis, heriditary spherocytosis, autoimmune hemolytic anemia, G6PD deficiency, and hemolytic anemia of uncertain origin.

Materials. NADP, NADPH, ADP, ATP, aspartic acid, α-ketoglutaric acid, phosphoenolpyruvate, glucose-6-phosphate, malic dehydrogenase, and lactic acid dehydrogenase (rabbit muscle) were from Sigma Chemical Co., St. Louis, Mo. 3-Phosphoglyceraldehyde, 3-phosphoglycerate kinase, and glyceraldehyde-3-phosphate dehydrogenase were from California Corporation for Biochemical Research, Los Angeles, Calif. Bovine serum albumin was from Hyland Laboratories, Los Angeles, Calif. All other chemicals were of reagent grade.

Hematologic indices. Blood cell counts and hemoglobin content were determined with a Coulter model S electronic particle counter (Coulter Electronics, Inc., Hialeah, Fla.). Leukocyte counts were usually also carried out manually. Reticulocytes were assessed by standard techniques (7). Spiculated cells were assessed by counting 500 cells suspended in a pH 7.4 solution consisting of isotonic phosphate-buffered saline plus 1% albumin. Only small, dense cells with prominent spicules were classified as spiculated (see Fig. 8, panel D).

In vivo studies of erythrocyte turnover. Survival of ⁵¹Cr-labeled autologous erythrocytes was measured according to the method of Read, Wilson, and Gardner (8). Ferrokinetic and ⁵⁹Fe in vivo organ radioactivity studies were carried out as described by Elminger, Huff, Tobias, and Lawrence

TABLE 1

Patients with Pyruvate Kinase Deficiency—Clinical Data

Patient	Age	Sex	Hemo-globin	Reticu-locyte	PK*	Spleen
	yr		g/100 ml	%		
M. P.	8	M	9.1	38	0.33	−
J. P.	1	M	9.2	41.2	0.66	−
C. D.	2	F	8.4	54	1.02	−
L. L.	10	F	8.9	65	0.99	−
S. L.	3	M	8.1	39.6	1.27	−
J. D. P.	26	M	13.8	8	0.47	+

* μmoles/liter per min per 10¹⁰ RBC. Normal = 2.0–2.73.

(9). The turnover of bilirubin in plasma was measured after intravenous infusion of tritiated bilirubin by the methods described by Berk, Howe, Bloomer, and Berlin (10).

Incubations. The blood samples were centrifuged for 10 min at 400 g. The plasma and buffy coat were removed by aspiration, and the plasma was then centrifuged for 15 min at 4500 g, the supernatant being put aside for later use in some experiments. The erythrocytes were thrice washed in Krebs-Henseleit buffer, pH 7.4, to which had been added glucose (final concentration 10 mmoles/liter) and bovine serum albumin (final concentration 1 g/100 ml). Any visable leukocytes were removed by aspiration, but care was taken to preserve the reticulocyte-rich top layer of erythrocytes. The washed erythrocytes were then suspended in a fresh aliquot of the washing buffer at a hematocrit of 20–30% and added to stoppered 10- or 25-ml Erhlenmeyer flasks for incubation. Additions such as NaCN, EDTA, or fluoride were made after adjustment of pH to 7.4 in small volumes of isotonic saline or in buffer. A portion of the erythrocyte suspension was retained for blood cell counts and microhematocrit. Perchloric acid filtrates were obtained on this fraction to assess levels of metabolites before incubation.

The flasks were incubated at 37°C and at 60 oscillations per min. pH was measured frequently with a model 135 A pH meter equipped with an Ingold microelectrode (Instrumentation Laboratory Inc., Watertown, Mass.). By appropriate gassing of flasks with mixtures of air and 5% CO₂, it was possible to maintain pH at 7.45 ±0.1. For incubations in an atmosphere of nitrogen, a continuous flow of nitrogen and 5% CO₂ at 25–50 cc/min was bubbled through water, heated to 37°C, and passed through each flask. No. 15 gauge needles were thrust through the rubber flask stopper to provide inlet and outlet for the gas. Up to four flasks could be connected in parallel to the gas supply and gassed simultaneously. Blood samples were obtained by piercing the flask stopper with a No. 20 spinal needle attached to a syringe. After incubation in nitrogen, the blood pH was found to range between 7.35 and 7.48.

For measurement of metabolites, perchloric acid extracts were prepared. ATP (11), lactate (12), and 2,3-diphosphoglycerate (13) were measured according to the cited methods on aliquots of the neutralized perchloric extract. Appropriate control studies showed that small amounts of cyanide carried over into the assays in experiments in which this agent was used did not influence the assays.

Enzymes were measured on dilute hemolysates prepared by osmotic lysis of erythrocytes. Before lysis, leukocytes were further removed by filtration through a double layer of Whatman filter paper No. 2. Control studies showed that reticulocytes were not retained by the filter. After lysis, the hemolysates were quickly frozen and thawed once to

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insure complete hemolysis and centrifuged for 10 min at 4500 g to remove membranes. Pyruvate kinase (14) and G6PD (15) were measured at 37°C utilizing minor modifications of the methods cited. Hemolysates were made on cyanide-treated blood in some experiments. Control studies showed that buffered cyanide solutions had no influence on the enzyme assays employed.

Oxygen consumption. Heparinized venous blood from patient with reticulocytosis was passed through dacron wool (Leuko-Pak, Fenwall Laboratories, Inc., Morton Grove, Ill.). This procedure reduced platelets to fewer than 2000/mm³ and leukocytes to less than 1500/mm³. Next, the cells were thrice washed in 0.1 M Krebs-Ringer phosphate buffer, pH 7.4, enriched with 10 mM glucose, and resuspended in the same buffer at a hematocrit of 30%. Red blood cell, nucleated red blood cell, and leukocyte counts were obtained, and the percentage of reticulocytes was determined.

The rate of oxygen consumption was determined with a Clark oxygen electrode (Yellow Springs Instrument Company, Yellow Springs, Ohio) equipped with a scale-expansion recorder. The incubation mixture was 3 ml of reticulocyte-rich blood in air. The rate of oxygen consumption was determined at 37° for at least 2 min. The oxygen consumption of the residual leukocytes was found to be inconsequential.

Measurement of cations. Incubations were performed in Krebs-Henseleit buffer as described earlier. At intervals, supernatant samples were obtained and analyzed for K^+ and Na^+ content (16). Duplicate results for duplicate flasks were averaged and plotted against time. Potassium loss per liter of cells was calculated by dividing the potassium lost per unit of time by initial hematocrit. No correction was made for trapped plasma in determining the hematocrit.

Blood rheology. Heparinized blood was prepared as described for measurement of oxygen consumption except that erythrocytes were not washed with buffer, but instead were resuspended in autologous plasma. For experiments with acidified blood, sufficient lactic acid was added to bring the pH below 7. Whole blood viscosity was measured at low shear rates (less than 20 sec⁻¹) in a GDM viscometer (Instrumentation Laboratory, Massachusetts Institute of Technology, Cambridge, Mass.) and at higher shear rates (greater than 11.5 sec⁻¹) in a Wells-Brookfield cone-plate viscometer model LVT (Brookfield Engineering Laboratories, Inc., Stoughton, Mass.), according to methods previously described (17). For filtration studies, 2% suspensions of erythrocytes in autologous plasma were prepared. The time required for 2 ml of such a suspension under 15 cm of water pressure at 37°C to pass through an 8 μ Millipore filter was measured and the rate of flow determined, as previously described by Murphy (18).

Ultracentrifugation of cyanide-treated PK cells. After removal of plasma erythrocytes were thrice washed in Krebs-Henseleit buffer enriched with 10 mM glucose and 1% albumin, then suspended in the same buffer, and filtered twice through double layers of Whatman filter paper No. 2 to remove leukocytes (19). The buffer was removed by centrifugation and the filtered erythrocytes resuspended in autologous plasma. An aliquot was then incubated with no additive or with 5 mM NaCN for 2 hr at 37°C in a shaking water bath to induce spiculated cell formation. After incubation the blood was ultracentrifuged at 100,000 g for 1 hr at 4°C. The top 20% of the column of centrifuged erythrocytes was removed by aspiration and the bottom 20% by gravity. Blood cell counts were obtained on each layer. The erythrocytes were then thrice washed in cold normal

saline and the top layer cells passed for a third time through filter paper as described earlier. After this filtration, fewer than 1 leukocyte per 600 erythrocytes remained, and differences in leukocyte count between top and bottom layers were negligible. Assays for pyruvate kinase and G6PD activities were then carried out in duplicate on a portion of the saline-washed cells. The remainder of the erythrocytes were then washed twice in a buffer which contained 5 mM adenosine, 10 mM glucose, 1% albumin, 2 mM magnesium, 20 mM phosphate, 135 mM potassium, 15 mM sodium, 4 mM sulfate, and 95 mM chloride at pH 7.5 The washed cells were resuspended in this buffer at a hematocrit of approximately 10%. After blood cell counts, microhematocrit, and initial perchloric acid extracts were obtained, the blood was added to flasks and incubated for 2 hr. Perchloric acid extracts were again obtained.

RESULTS

Turnover of newly formed PK-deficient reticulocytes

Radioisotope studies of tritiated bilirubin turnover, survival of autologous ⁵¹Cr-labeled erythrocytes, and ferrokinetics were obtained in an adult male (J.D.P.) with splenomegaly-associated with hemolytic anemia and erythrocyte PK activity of only 0.47 U/10¹⁰ RBC with no evidence for altered substrate affinity. The results may be seen in Table II and Fig. 1. Although J.D.P. was chronically jaundiced and exhibited persistent reticulocytosis of 5–8%, the mean life span of his

TABLE II
Erythrocyte Pyruvate Kinase Deficiency—Patient J. D. P.

	Patient J. D. P.	Normal*
Clinical data‡		
Hemoglobin (g/100 ml)	12.9–14.5	16 ±2
Reticulocytes (%)	5.3–8.0	3.0
Indirect bilirubin (mg/100 ml)	2.45	0.7
³H bilirubin turnover		
Bilirubin production		
(mg/kg per day)	15.4	3.8 ±0.6
RBC life span (days)	20	100
Autologous ⁵¹Cr RBC survival		
$t_{\frac{1}{2}}$ (days)	26, 33	26–35
RBC life span (days)	100	100
Ferrokinetics		
Plasma Fe clearance (t½, min)	26	85–110
Fe uptake (%)	54	85–100
Plasma Fe turnover		
(mg/kg per day)	1.69	0.4–0.45
RBC life span (days)§	27.6	100 ±20

* Values are those found for normal adults in our laboratory. Normal values cited for bilirubin turnover were obtained in 19 normal individuals studied at the National Cancer Institute by Dr. Joseph Bloomer and his coworkers. (Personal communication.)
‡ Results are the range of values observed over 2 yr of study.
§ Calculated from plasma and red cell iron turnover.

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circulating erythrocytes as estimated by the ^{51}Cr method was within the normal range. Furthermore, body scanning revealed minimal or no splenic trapping of J.D.P. ^{51}Cr-labeled circulating erythrocytes. These results were not consistent with the clinical evidence of accelerated erythrocyte destruction.

Ferrokinetics, assessed simultaneously with the second ^{51}Cr survival study, revealed a plasma clearance of ^{59}Fe and plasma iron turnover which were more than 3 times the normal rate. The mean red cell survival time, calculated from plasma and red cell iron turnover, was approximately 27 days. Iron uptake by peripheral erythrocytes revealed a rapid initial phase followed by slow attainment of a maximum incorporation of 54% of the dose in 22 days, a result consistent with recycling of iron after rapid hemolysis of newly formed cells either in the marrow or in the spleen and liver (20).

Organ count rates (Fig. 1) indicated the fate of the short-lived ^{59}Fe-labeled cells. After infusion, labeled iron was rapidly taken up by the bone marrow and incorporated into erythrocytes, many of which were promptly released into the circulation. Splenic and liver radioactivity was high initially, reflecting the blood flow through these organs. As radioiron left the plasma and entered the marrow, radioactivity over the liver and spleen fell. In the first 3 days postinfusion splenic radio-
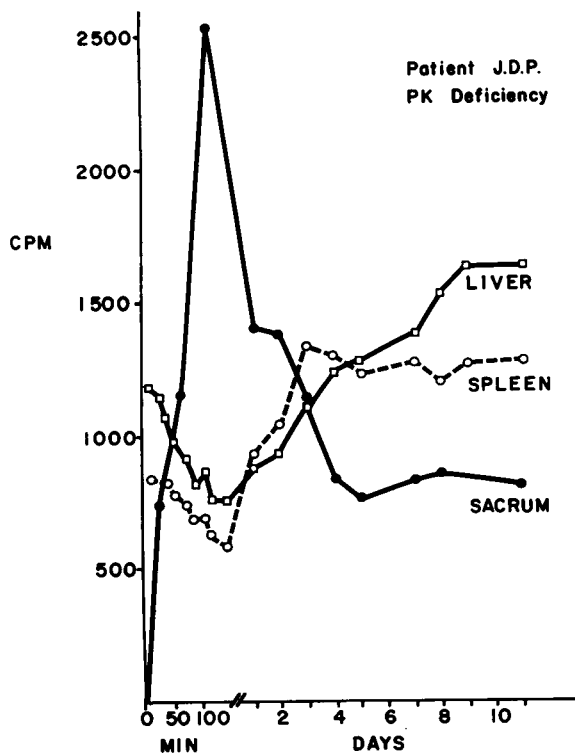
activity rapidly increased, clearly indicating sequestration of a proportion of newly formed erythrocytes. Similar sequestration began slightly later in the liver, but eventually counts over the liver exceeded those over the spleen. Persistence of sacral radioactivity was presumably due to recycling of iron from rapidly destroyed cells. Together, these results indicate that an unknown but sizable proportion of newly formed J.D.P. cells were sequestered in the reticuloendothelial system and promptly destroyed. The remainder survived very well despite their considerable enzyme deficiency and were sufficient in number to maintain hemoglobin levels at 13–14 g/100 ml in the peripheral blood.

The daily turnover of bilirubin, measured by intravenous injection tritiated bilirubin, provided further confirmation of these conclusions since the value was almost 5 times normal. Mean red cell life span calculated from the daily rate of bilirubin production was 20 days, a figure closely resembling that obtained from the plasma iron turnover. The discrepancy between these estimates of red cell survival and that obtained from ^{51}Cr-labeled circulating erythrocytes indicated the presence of a large pool of sequestered, short-lived cells. Because such cells presumably circulated only briefly or were destroyed in the marrow, they were largely unavailable for ^{51}Cr labeling. That low levels of pyruvate kinase need not dictate the survival of mature erythrocytes was additionally demonstrated by therapeutic transfusion of J.D.P. cells (PK = 0.47 μmoles/min per 10^{10} RBC) into C.D. (PK = 0.9 μmoles/min per 10^{10} RBC), who at the time exhibited marked splenomegaly anemia (hemoglobin 5.9 g/100 ml), and reticulocytosis (12.6%). Isologous survival of J.D.P. cells in C.D. was only modestly reduced (t$\frac{1}{2}$ ^{51}Cr, 18 days), whereas the autologous survival of C.D. cells was markedly shortened (t$\frac{1}{2}$ ^{51}Cr, 7 days).

In vitro studies

Glycolysis in PK-deficient reticulocyte-rich blood. Lactate production by PK-deficient and by control reticulocyte-rich blood is shown in Table III. In PK deficiency, lactate production was only one-fourth that found in controls. When oxidative phosphorylation was inhibited by cyanide or by anoxia, lactate production by PK-deficient blood rose slightly, but remained significantly (P 0.001) below control levels. Assuming that immature PK-deficient erythrocytes resemble control erythrocytes in their metabolic requirements, these results demonstrate the inability of glycolysis to fulfill such metabolic needs. Control blood, fully capable of adequate glycolysis for energy requirements, exhibited a modest increase in lactate production upon incubation in the presence of cyanide. In separate experiments, it was shown that lactate production by the few residual

FIGURE 1 ^{59}Fe organ scan. Patient J. D. P.

TABLE III

Lactate Production* by Reticulocyte-Rich Blood

	Number	% Reticulocytes	No additives	5 mM CN⁻	N₂
PK-deficient	5	23.6–58.4	1.91 ±0.65	2.92 ±0.59	2.96 ±0.31
Control	7	4–54	8.58 ±0.96	9.44 ±1.22	—

All incubations were carried out at 37°C, pH 7.4 ±0.1, in the presence of 10 mM glucose.

* mmoles/liter of cells per hr ±1 SD.

white cells in the incubated blood would not appreciably alter the reported erythrocyte lactate production rates.

ATP stability. The ATP concentration of PK-deficient erythrocytes did not differ significantly from control values, as shown in Table IV. Incubation of PK-deficient blood, however, usually resulted in a fall in ATP concentration, whereas control ATP concentration remained unchanged. Mitochondrial inhibition induced by cyanide produced a profound decline in ATP in PK-deficient blood, whereas only a modest fall occurred in control blood. Fig. 2 depicts the rapidity of the observed decline in ATP. Within 40 min, base line values were reached. ATP also declined when PK-deficient blood was incubated under nitrogen, which indicates that this was not a unique effect of cyanide. These observations demonstrate the importance of oxidative phosphorylation to the PK-deficient reticulocyte for synthesis of ATP. They further emphasize the unique susceptibility of such cells to inhibition of oxidative phosphorylation, an observation first made by Keitt (2).

Oxygen consumption by PK-deficient reticulocytes. Oxygen consumption was considerably increased in PK-deficient blood relative to that of reticulocyte-rich control blood (Table V). If the lesion of PK deficiency was simulated in control reticulocyte-rich blood by the addition of fluoride, which is known to inhibit enolase (21), an increase in oxygen consumption was observed. This indicated that oxidative phosphorylation pathways

were potentially available in the control cells though they were normally little utilized.

As noted above the PK-deficient cell is dependent in part upon respiration. Its ATP content is vulnerable to inhibitors of oxidative phosphorylation such as hypoxia or cyanide. That such sensitivity is of physiologic importance is shown in Fig. 3 which demonstrates the relation between available oxygen and oxygen consumption by the reticulocyte-rich blood of two PK-deficient patients. At the Po₂ of venous blood, oxygen consumption entirely disappeared. Prolonged exposure to venous Po₂ levels in vivo would presumably also suppress oxidative metabolism upon which the PK-deficient reticulocyte depends.

Ion and water permeability of PK-deficient reticulocytes. When oxidative phosphorylation was unavailable to PK-deficient reticulocytes, profound losses of intracellular potassium occurred. In the experiments shown in Fig. 4, either cyanide or hypoxia was used to inhibit oxidative phosphorylation. After a short lag period during which cellular ATP was depleted (Fig. 2), a rapid efflux of intracellular potassium ensued. Within a few hours more than half of all intracellular potassium was lost from the cell. In contrast, control reticulocyte-rich cells incubated with cyanide lost no more than 2 mEq of potassium per liter of cells per hr. PK-deficient cells incubated without cyanide exhibited a modest potassium loss of several milliequivalents per hour as previously reported (19, 22). The striking loss of potassium induced by cyanide in PK deficient blood could also be

TABLE IV

ATP Stability of Incubated Reticulocyte-Rich Blood

				ATP*		
					Postincubation‡	
	Number	Reticulocytes	Preincubation	No additives	5 mM CN⁻	N₂
		%		*mmoles/liter of cells*		
PK-deficient	5	28–60.8	1.56 ±0.37	0.99 ±0.41	0.47 ±0.09	0.49 ±0.09
Control	5	11–54	1.49 ±0.32	1.63 ±0.25	1.15 ±0.31	—
				$P < 0.02$	$P < 0.01$	

* Mean ±1 SD.

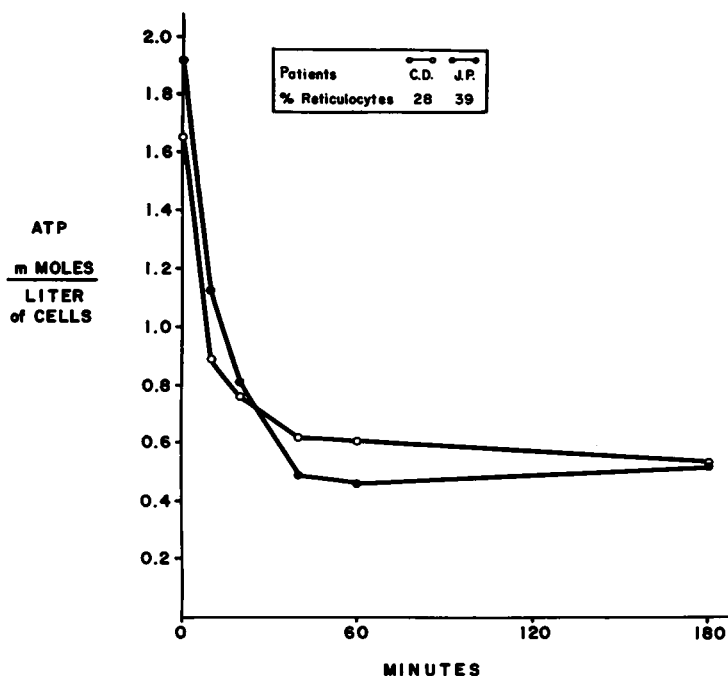‡ All incubations carried out for 3 hr at 37°C, pH 7.4 ±0.1, in presence of 10 mM glucose.

FIGURE 2 Effect of 5 mM CN⁻ on ATP stability of incubated PK-deficient blood. Temperature = 37°C; 10 mM glucose.

induced by gassing with nitrogen. Due to the low gas flow rates employed, equilibration of blood with nitrogen required 60–90 min delaying the onset of heightened potassium efflux. Once begun, however, the rate of potassium loss produced by hypoxia equaled that caused by cyanide. Only a small portion of the massive potassium loss was due to failure of the membrane ATPase pump secondary to ATP depletion since inhibition of the cation pump by 10^{-4} M ouabain resulted in net potassium losses from the erythrocytes of M.P. and C.D. of only 3.75 and 3.82 mEq/liter of cells per hr. These data indicated that a profound membrane abnormally was induced by ATP depletion allowing the migration of potassium from the cell.

Cyanide induced potassium loss from PK-deficient cells could be reduced greatly by previous addition of EDTA to the medium. Addition of 10^{-2} M ATP, however, had no effect on potassium loss nor did incubation in either a calcium-free or a calcium- and magnesium-free buffer. EDTA had no effect on the inhibition of oxidative metabolism produced by cyanide or nitrogen in the erythrocytes of C.D. or M.P., nor did it prevent ATP depletion. The possibility that cyanide itself might have had an additional direct effect on the membrane was unlikely since incubation in a nitrogen atmosphere, without added inhibitors, was also capable of producing extreme potassium losses from susceptible cells. Furthermore, when the erythrocytes of M.P. were incubated in the simultaneous presence of both cyanide and nitrogen (shown in Fig. 4), EDTA was still capable of preventing excess loss of potassium.

TABLE V

Oxygen Consumption by Reticulocyte-Rich Blood

	Number	Reticulocytes	Oxygen consumption*		
			No additives	1 mM CN⁻	10 mM F⁻
		%	μl O₂/10⁹ reticulocytes per hr		
PK-deficient	5	26–70.2	3.75 ±1.55	0.15 ±0.3	—
Control	5	12.4–39	0.56 ±0.5	0.04 ±0.03	1.89 ±0.82
			$P < 0.01$	NS	

* Mean ±1 SD. Conditions of study as described in text.

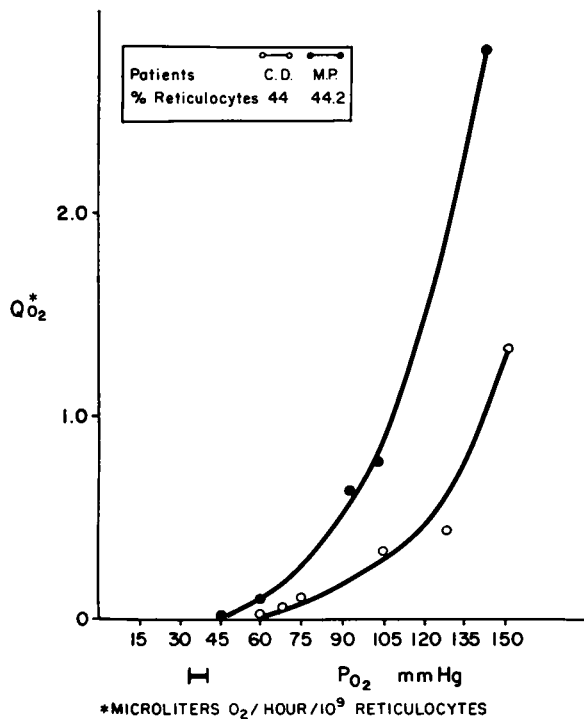| Patients	C.D.	M.P.
% Reticulocytes	44	44.2

FIGURE 3 Influence of P_{O_2} on oxygen consumption by PK-deficient reticulocytes. The normal range for venous P_{O_2} is indicated by the solid bar.

*MICROLITERS O_2/HOUR/10^9 RETICULOCYTES

During incubation of PK-deficient blood with cyanide or in nitrogen, net efflux of potassium initially exceeded influx of sodium. Similar cation shifts have been reported in stored, ATP-depleted, normal erythrocytes (23). In the cyanide experiments shown in Fig. 4, the net sodium gained during the first 3 hr of incubation averaged 30.1 mEq/liter of cells, whereas net potassium loss during the same period averaged 51.6 mEq/liter of cells. Therefore, during this period an over-all loss of intracellular cations occurred, necessarily accompanied by obligate osmotic losses of water. The resulting loss in cell volume was reflected by a 15–17% fall in hematocrit. Hemolysis during the incubation period was found to be less than 1% when sequential measurements of supernatant hemoglobin were made. The observed cation changes were, therefore, largely prelytic. The appearance of the shrunken cells produced by incubation is shown in Fig. 5. Two populations of erythrocytes were easily identified; one apparently normal and the other markedly shrunken, distorted, and spiculated.

The rheology of PK-deficient reticulocyte-rich blood. In an attempt to understand the fate of shrunken spiculated PK-deficient cells in vivo, the rheology of such blood was studied in vitro. As shown in Fig. 6, the whole blood viscosity of PK-deficient reticulocyte-rich blood closely resembled control values at all shear rates stud-

ied. Incubation with cyanide produced little change in the viscosity of reticulocyte-rich control blood but increased significantly (P 0.01) the viscosity of PK-deficient blood. The studies with cyanide shown in Fig. 6 were carried out on the blood of four patients at high shear rates where the degree of cell deformability or rigidity has a major influence on viscosity (24). At lower shear rates where viscosity is largely determined by the aggregation tendencies of cells (25), studies were obtained on only one patient (C.D.). C.E.'s blood viscosity was significantly increased at low as well as at high shear rates.

Fig. 7 presents the results of millipore filtration studies. High reticulocyte control blood, both pre- and postsplenectomy, filtered rapidly and was not influenced by cyanide. Postsplenectomy PK-deficient blood filtered as well as control blood. A significant retardation of filtration was produced in PK-deficient blood by cyanide, however, and similar results were achieved by lowering the pH below 7. A modest reduction in filtration by acidification was also seen in three control blood samples.

Centrifugation studies. It was possible to obtain by centrifugation a fairly homogeneous population of the shrunken, dense, spiculated cells produced by cyanide. Table VI and Fig. 8 reveal the metabolic and morpho-
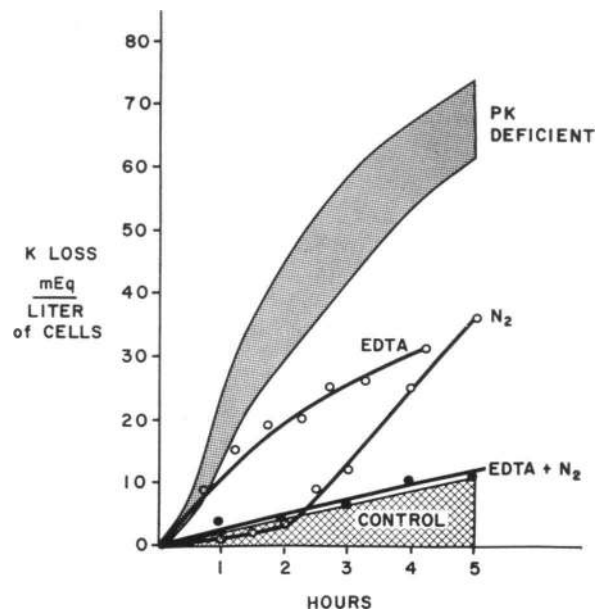

FIGURE 4 Effect of mitochondrial inhibition on potassium loss from PK-deficient blood. Shaded area: the range of eight studies in PK patients M. P., J. P., and C. D.; 5 mM NaCN. Cross hatched area: the range of five studies in three non-PK patients with reticulocytosis; 5 mM NaCN. Curve N_2, patient C. D.; 95% N_2 + 5% CO_2. Curve EDTA, patient C. D.; 5 mM NaCN and 5 mM EDTA. Curve EDTA + N_2, patient M. P.; 5 mM NaCN, 5 mM EDTA, and 95% N_2 + 5% CO_2.

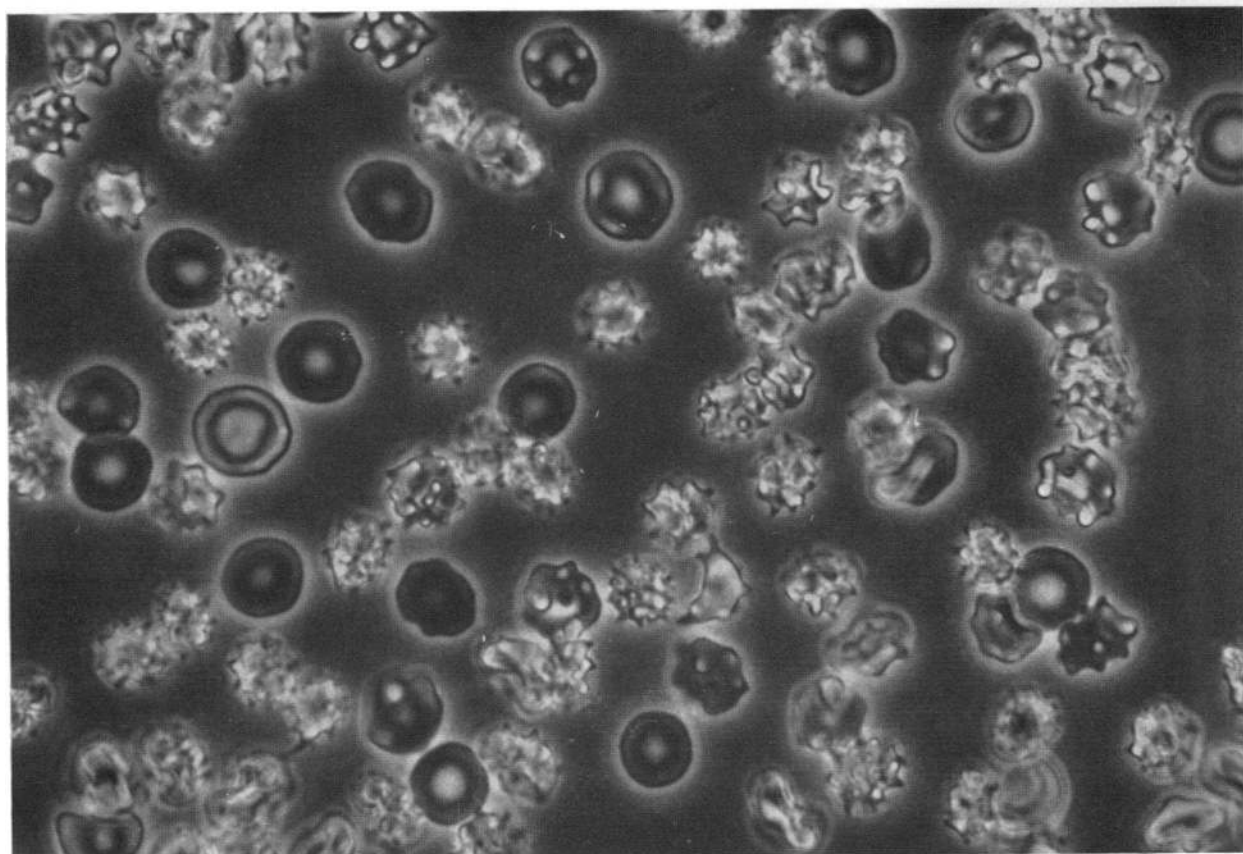694   *Mentzer, Baehner, Schmidt-Schönbein, Robinson, and Nathan*

FIGURE 5 Phase-contrast photomicrograph of PK-deficient blood (patient C. D.) after 2 hr exposure to 5 mM cyanide. × 6600.

logic characteristics of such cells. Reticulocytes predominated in the top layer of blood centrifuged without exposure to cyanide, and in the bottom layer of cyanide inhibited blood. These bottom layer reticulocytes were shrunken and spiculated. G6PD activity, an index of mean cell age, indicated that the bottom layer of cyanide-treated PK blood was younger than the top, a result in keeping with the reticulocyte counts. In contrast, PK activity was higher in the top layer of cyanide-treated blood, which suggests that the shrunken bottom layer reticulocytes were particularly deficient in PK. When the various layers obtained by centrifugation were incubated in a substrate-enriched high potassium buffer which restored cell volume, the bottom layer of spiculated cells were found to have a very low rate of lactate production and a high 2,3-DPG. In fact, the cyanide-treated bottom layer cells had the same 2,3-DPG content as the cyanide-free top layer cells, which indicates that the metabolic impairment was much greater in young cells. However, those young cells which remained in the top layer despite exposure to cyanide had much lower 2,3-DPG levels, reflecting their greater glycolytic capabilities.

DISCUSSION

Although young PK-deficient cells obtained by centrifugation exhibit more PK activity than older cells (26), both young and old cells are deficient in activity when compared with normal erythrocytes. PK deficiency, therefore, differs from the G6PD deficiency observed in Negroes in which unduly rapid decay of initially normal enzyme activity occurs. Because PK is normally not rate limiting in erythrocytes (14), a deficiency of this enzyme can be associated with levels of glycolysis normal for mature erythrocytes (27). On the other hand, normal immature erythrocytes, largely dependent upon glycolysis despite the presence of mitochondria, exhibit increased rates of glycolysis compared with mature cells which suggests that their requirements for ATP renewal are greater. It should not be surprising, therefore, to find that young PK-deficient cells are more severely compromised by their deficiency than are older cells.

Although only a single individual was studied, the clinical and radioisotope studies of J.D.P. strongly support the conclusion that young cells are particularly liable to destruction in PK deficiency. Despite normal
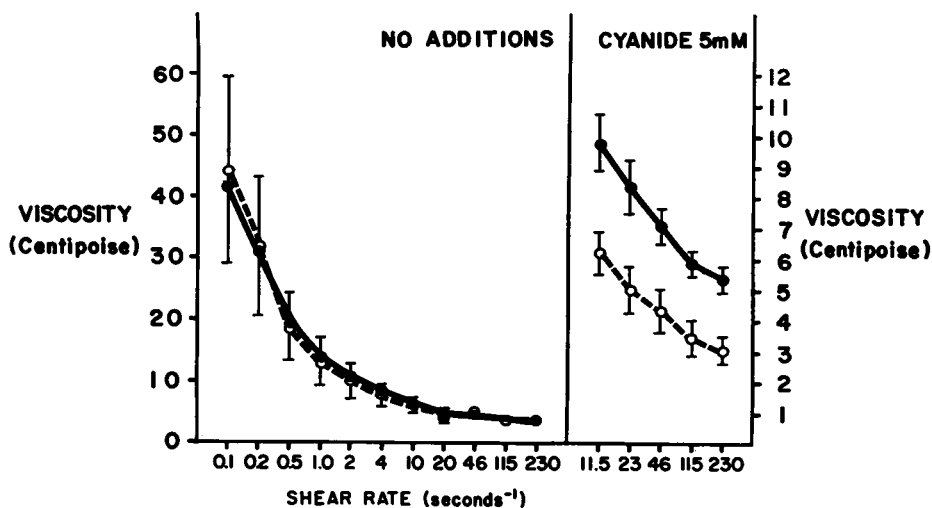
Reticulocyte Destruction in PK Deficiency 695

FIGURE 6 Effect of 5 mM cyanide on whole blood viscosity of PK-deficient blood. Temperature = 37°C; Hematocrit = 40%. ●——● PK deficient; ○——○, control. Values represent mean ±1 SD.

survival of his ^{51}Cr-labeled peripheral red cells (a finding reported previously in other PK-deficient patients [28, 29], and additionally suggested for at least some of the cells in severely anemic PK patients by their biphasic ^{51}Cr survival curves), there was clear cut evidence from ^{59}Fe and bilirubin-^{3}H studies of destruction of a large proportion of the newly formed erythrocytes of this patient. The ^{59}Fe organ scans showed that the spleen and liver were major sites of death of the newly formed cells and additionally suggested, as had previous studies, that initial splenic sequestration was followed by terminal destruction in the liver.

The in vitro studies were designed to investigate the metabolic basis of reticulocyte vulnerability in PK deficiency. Keitt initially recognized the dependence of PK-deficient reticulocytes on mitochondrial function, observing that cyanide largely abolished ATP stores in deficient cells but had little effect on normal reticulocytes (2). Our studies provide further support for this concept by demonstrating the high rate of oxygen consumption by PK-deficient reticulocytes. Cyanide-induced ATP loss was remarkably rapid in affected erythrocytes, presumably as a result of the increased ATP requirements of immature cells. The consequences of ATP loss in PK-deficient cells were similar to those observed in fluoride-treated red cell ghosts by Lepke and Passow (30), and were also similar to some of the findings of Weed, LaCelle, and Merrill (31). PK-de-
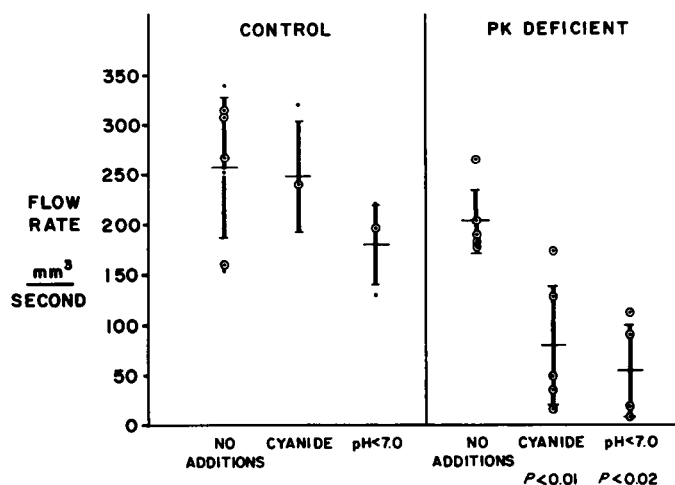

FIGURE 7 Effect of 5 mM cyanide and low pH on filterability of PK-deficient blood. ⊙, splenectomy; ●, no splenectomy.

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ficient cells became extremely leaky to cations, but lost potassium much more rapidly than they gained sodium. The K loss was retarded when EDTA was present in the medium, but was unaffected by removal of calcium and magnesium from the buffer. It seems likely, therefore, that the loss of ATP in PK-deficient cells permitted membrane calcium to react unfavorably with certain other membrane constituents, an event which greatly disturbs the normal membrane barrier to the passage of univalent cations. The nature of this interaction of membrane Ca^{++} to membrane permeability is entirely unknown. Weed et al. (31) have found that internal ATP loss leads to gross stiffening of the membrane itself. We did not evaluate the stiffness of cyanide-treated PK-deficient membranes, but the cells themselves became much more viscous and rigid in the presence of cyanide. This was presumably a consequence of cell dehydration as well as the effects of ATP depletion on the membrane itself. The result was decreased filterability and increased viscosity of the shrunken PK cells.

Although cyanide was utilized in many of our studies, it was also shown that an atmosphere of nitrogen resulted in ATP loss, potassium loss, cell shrinkage, and increased cell viscosity. This provided further evidence for the importance of respiration in the maintenance of PK cellular integrity. In addition, it was noted that respiration in PK-deficient cells ceased at a Po_2 of between 45 and 69 mm Hg, levels just above the Po_2 of venous blood. Finally, as predicted from the studies of Murphy (18), incubation of PK-deficient cells in buffers below pH 7.0 resulted in markedly decreased filterability. Therefore, it seems clear that a prolonged encounter with the static, anoxic, and acidic venous system in the splenic pulp (32), would lead in vivo to the unfavorable consequences that we have described in vitro.

The final set of experiments reported in this paper provided further confirmation of the vulnerability of PK-deficient reticulocytes to inhibition of respiration. When the shrunken, spiculated cells produced by cyanide were collected by centrifugation, they proved to be largely reticulocytes. The PK activity of the spiculated, bottom fraction was particularly low relative to the high percentage of reticulocytes present, which suggests that there may be heterogeneity of the PK content of newly formed reticulocytes. Those with exceptionally low PK activity would, by virtue of their extremely low glycolytic rates, be highly susceptible to mitochondrial inhibition. Reincubation of these cells in a high potassium medium permitted them to regain water and potassium, presumably by passive diffusion. Despite the use of adenosine as substrate in order to bypass the ATP-dependent hexokinase and phosphofructokinase reactions, the glycolytic rates of bottom layer cells were severely limited, and their 2,3-DPG levels were markedly increased.

We conclude from all these data that PK reticulocytes may in fact vary in their levels of PK activity. As the PK activity in a reticulocyte approaches a critically low level, the ATP production of the cell becomes dependent upon mitochondrial metabolism. When such cells emerge from the marrow, they may be detained by the spleen. There, the Po_2 of the splenic pulp blood is insufficient for the maintenance of reticulocyte mitochondrial metabolism. ATP levels fall rapidly, and the cell loses both potassium and water. It becomes dehydrated, viscous, and spiculated and is either destroyed in the spleen or, if it emerges from that organ is so compromised that it is soon destroyed in the liver. Some

TABLE VI
Centrifugation of Pyruvate Kinase–Deficient Blood

	Normal blood	No cyanide		Cyanide	
		Top	Bottom	Top	Bottom
Mean corpuscular volume, μ^3					
Before incubation		125	115	119	109
After incubation		126	119	120	118
Reticulocytes, %		60	24	21	47
Spiculated cells, %		6.6	10.8	17.8	93.4
PK activity, $\mu moles/liter$ per min per 10^{10} RBC	2.0–2.7	0.5	0.27	0.69	0.25
G6PD activity, $\mu moles/liter$ per min per 10^{10} RBC	2.43–3.34	7.89	6.18	5.31	5.90
Lactate production, $mmoles/liter$ of cells per hr	8.58 ±0.96*	2.8	2.0	4.0	0.45
2,3-DPG, $mmoles/liter$ per 10^{12} RBC	0.46 ±0.04				
Before incubation		2.0	1.98	1.62	2.18
After incubation		2.10	1.90	1.61	2.10

PK-deficient cells were incubated with or without 5 mM NaCN for 2 hr at 37°C. They were then centrifuged at 100,000 g for 1 hr. The top and bottom fractions were then incubated again in a high potassium, glucose, and adenosine-containing buffer. See Methods for details.
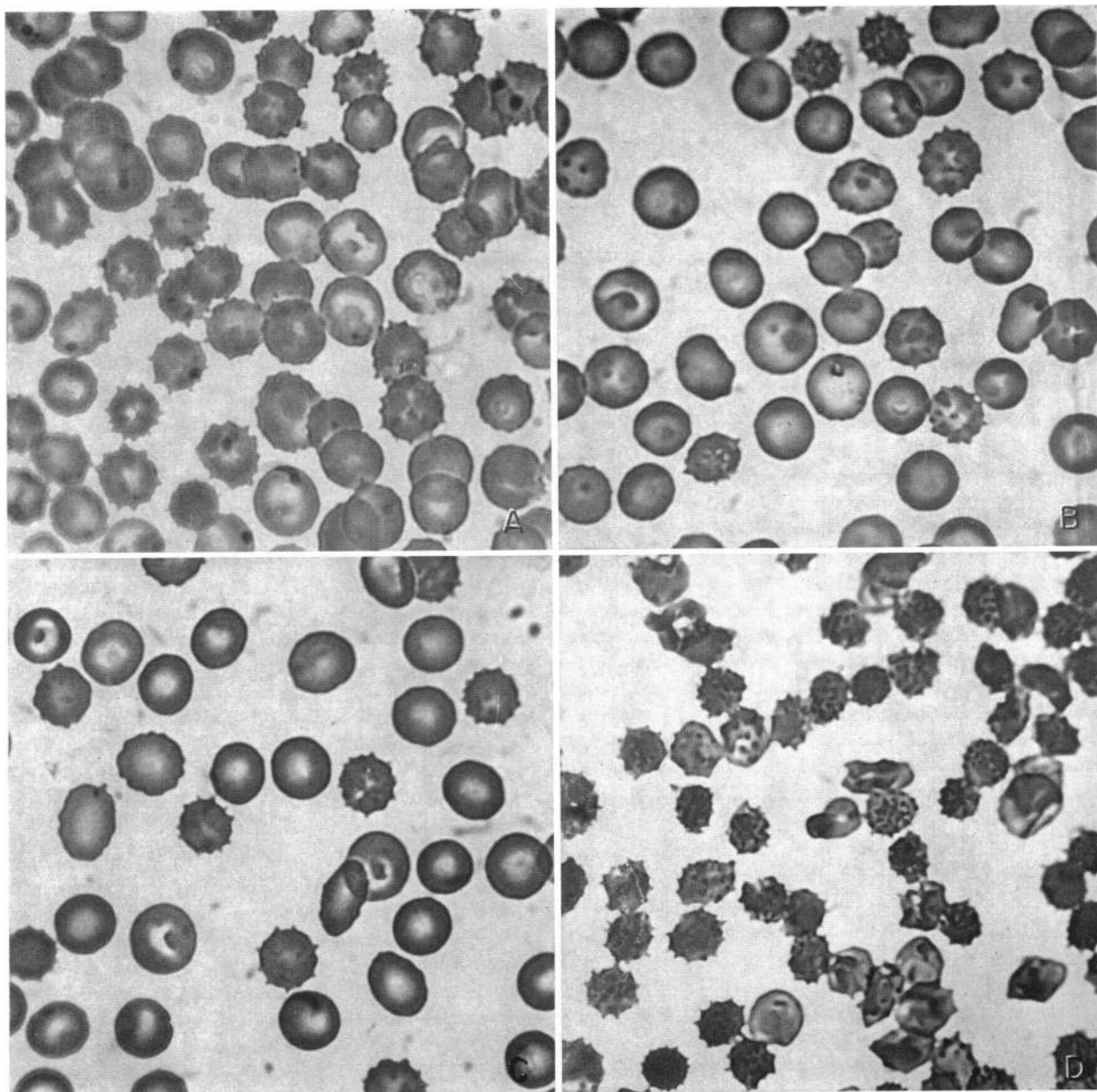* Values for reticulocyte-rich blood.

FIGURE 8 Appearance of ultracentrifuged PK-deficient blood (patient C. D.). A, top, and B, bottom layers of blood not exposed to cyanide. C, top, and D, bottom layers of blood exposed to 5 mM cyanide (Wright's stain). × 5000.

reticulocytes survive to maturity, either by virtue of their greater PK activity or because, purely by chance, they have avoided prolonged or repetitive exposure to an environment, such as is found in the spleen, where inhibition of cell respiration may occur. As PK reticulocytes mature, they lose mitochondria, but their demands for ATP also decrease. Their decline in ATP requirements permits the mature cells of some patients to circulate with very low PK levels. This is evidenced by

J.D.P. whose ^{51}Cr red cell survival was normal but whose PK level was lower than four of the five patients who had intense hemolysis and who required splenectomy.

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