Comparison of Red Cell Creatine Level and Reticulocyte Count in Appraising the Severity of Hemolytic Processes

By Jorg Fehr and Margarethe Knob

In seeking a sensitive indicator for quantitative assessment of hemolytic disease, we found a close dependence of red cell creatine level on cell age. Studies in 21 patients with steady-state hemolysis showed high correlation \( r = 0.89, p < 0.001 \) between reticulocyte counts and red cell creatine levels. Excluding elevation of the creatine level as a variable epiphenomenon of increased erythropoietic activity, density separation of normal red cells revealed distinctly higher creatine levels in younger cells. The reticulocyte counts and creatine levels as quantitative predictors of hemolytic processes were compared: in severe hemolytic anemias \( (T_{50}Cr < 11 \text{ days}) \), erythrocyte survival \( (T_{50}Cr) \) correlated well with creatine levels \( r = -0.86, p < 0.01 \) and, to a lesser degree, with reticulocyte counts \( r = -0.72, p < 0.05 \). In milder disease \( (T_{50}Cr > 11 \text{ days}) \), however, no correlation existed between reticulocyte counts and \( T_{50}Cr \), whereas the creatine levels correlated closely with \( T_{50}Cr \) \( r = -0.84, p < 0.001 \). Thus, on the basis of our regression equations, useful estimation of red cell survival may be obtained from single measurements of erythrocyte creatine.

The essential feature of a hemolytic disease is a reduction in the life span of the patient’s erythrocytes. Although the problem seems clear, no direct and simple measure is available for determining the presence of a hemolytic process. Diagnosis of a hemolytic disease usually requires careful analysis of the findings that pertain to red cell production and red cell destruction rates. The widely used laboratory tests aimed at detecting increased breakdown products of red cells allow at best a rough quantitative estimation of the intensity of the hemolytic process, and these tests are influenced by many factors. For instance, hyperbilirubinemia may be inappropriately increased by an accompanying liver dysfunction, the haptoglobin level may be raised unspecifically during an inflammatory process, and exogenous carbon monoxide resulting from smoking or air pollution may cause significant errors in the assessment of endogenous carbon monoxide production due to heme catabolism. Owing to the fact that these indices of increased breakdown of red cells are relatively imprecise, the intensity of a hemolytic process is assessed mainly by its compensatory increase in red cell production. The most readily available parameter for estimating red cell formation is the reticulocyte count, which usually reflects the degree of shortening of the red cell life span. The principal difficulty with this method results from the fact that reticulocyte counting is associated with high degree of statistical error, and the reticulocyte count reflects not only the rate of reticulocyte production but also the length of time such cells are identifiable as reticulocytes in the peripheral blood. Although numerous correction factors have been proposed that should lead to...
more accurate reflection of the erythrocyte production rate, it has not been demonstrated that diagnostic precision is improved thereby. Even the polychromatic "shift" red cells are often an unreliable index of premature red cell release. Moreover, in a large group of hemolytic anemias (the idiopathic immunohemolytic type) one-fourth of patients have been found to have normal reticulocyte counts. It can therefore be concluded that the reticulocyte count is an imprecise predictor of the rate of red cell destruction, from which red cell life span can be deduced. Because of obvious limitations of quickly estimating the severity of hemolytic processes, measurement of a direct variable of red cell age could undoubtedly provide valuable information. In 1967 Griffith and Fitzpatrick reported that the creatine content of red cells is a sensitive criterion of the mean age of the population. To study the clinical applicability and the value of red cell creatine measurements, we related mean erythrocyte creatine levels with direct determinations of red cell survival and compared creatine content and reticulocyte counts as quantitative predictors of red cell survival in different types of hemolytic anemias.

MATERIALS AND METHODS

Twenty-one patients with hemolytic disease were analyzed in this study (Table 1). Only patients with clear-cut hemolysis with a 18Cr-erythrocyte half-life (T1/2Cr) of 20 days or less were included. This upper limit was chosen after establishment of a 95% confidence range for T1/2Cr of 23–27 days obtained from red cell survival studies performed at our institution in 13 healthy male volunteers. A second prerequisite for patients to be included in this study was the necessity for fairly strict steady-state conditions. This means that the patients did not receive blood transfusions during the preceding month and throughout the period of the 18Cr-erythrocyte survival study and that their packed cell volumes changed not more than 4% during this time.

The types of hemolytic diseases in the 21 patients varied and included a homogeneous group of 9 patients with clinically and serologically typical features of autoimmune hemolytic anemia (AIHA). Of these, 7 patients had warm autoantibodies (4 patients had the idiopathic type, 2 patients had Hodgkin's

<table>
<thead>
<tr>
<th>Table 1. Hematologic, Red Cell Creatine, and Red Cell Survival Data for 21 Patients With Hemolytic Diseases</th>
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<tr>
<td>Patient</td>
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<td>A. P.</td>
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<td>F. I.</td>
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<td>J. F.</td>
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<td>Z. C.</td>
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<td>S. S.</td>
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<td>H. P.</td>
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*CV = Coefficient of variation (SD/mean) for the three to four determinations of reticulocytes and creatine, respectively, during each red cell survival study.
lymphoma, 1 patient had a histiocytic lymphoma); 2 patients had cold autoantibodies. Of the remaining 12 patients, 3 had hereditary spherocytosis, 3 had hypersplenic syndrome of unknown origin, 2 had Zieve's syndrome, 2 had hemolysis associated with chronic uremia, 1 had hereditary ovalocytosis, and 1 had idiopathic pancytopenia with hemolysis. The mean hematocrit of the group with AIHA was 25% (range 20%-29%); it was 33% (range 18%-46%) for the remaining group with other hemolytic diseases.

Labeling of red cells with $^{51}$Cr was carried out according to recommendations of the ICSH panel. ICSH method C, with whole-blood samples for labeling and removal of unbound chromium through addition of ascorbic acid, was used throughout the study. Measurements of $^{51}$Cr activity in blood were continued until at least half the radioactivity had disappeared from the circulation. In analyzing the data the red cell activity (with and without correction for elution) was first plotted against time on linear graph paper. If a straight line could be fitted to the data points, the line was obtained by a least-squares fitting procedure, and the $T_{\text{el}}$, the elution-corrected $T_{\text{M}}$, and the mean cell life span were determined. If a straight line did not fit the data well, the plotting and calculations were repeated on semilogarithmic paper. One of the two possibilities always resulted in a satisfactory linearization of the plot. The fitting procedure was performed by an experienced technician without knowledge of the red cell creatine data.

Blood was collected in EDTA for routine hematologic values. The red cells were counted with a Coulter Model S counter (Coulter Electronics, Hialeah, Fla.). Packed cell volumes were measured in microhematocrit tubes following centrifugation at 12,000 g for 7 min. Reticulocytes were stained at $37^\circ$C and counted on dry smears in at least 1000 cells. The mean of three to four determinations during a $^{51}$Cr-erythrocyte survival study was taken as the representative reticulocyte count for each patient. The normal values for reticulocyte counts in our laboratory are 0.4%–2.5% or, in absolute numbers, 17,000–125,000/ml (range $n = 85$).

For red cell creatine measurements 5 ml of blood were drawn into heparinized tubes and kept in the refrigerator for up to 7 days. No decrease in the creatine content was observed under such storage conditions. On the day of the creatine determination the tubes were centrifuged at 2000 g for 10 min, the plasma and the buffy coat were aspirated, and the red cells were carefully mixed. It was not necessary to wash the cells, since the plasma creatine concentration is too low to contribute significantly (less than 0.5 mg/dl of plasma, even after storage of the heparinized blood for 7 days at 4°C). Contaminating white cells and platelets can also be disregarded, since virtually no creatine was found in white-cell-enriched and platelet-enriched suspensions. One-tenth milliliter of packed cells was washed out into 0.9 ml of 0.1% saponin (w/v) in distilled water, and the creatine content was determined by the diacetyl-l-naphthol reaction adapted to the Technicon AutoAnalyzer. The mean of three to four different blood samples taken at the beginning, in the middle, and at the end of the $^{51}$Cr-erythrocyte survival study was used as the representative red cell creatine value for each patient. In order to translate the measured creatine values into quantities per diciliter of red cells, the results were multiplied by a correction factor of 1.087. This factor was based on microhematocrit measurements in 200 consecutive samples that showed a value of 92 ± 2.8% SD.

2,3-DPG was determined enzymatically by measuring the decrease in optical density at 340 nm due to NADH oxidation using the Sigma test kit. The normal range for 2,3-DPG in our laboratory is 11.7–16.9 μmoles/g hemoglobin.

Red cells of 9 normal individuals were separated according to their densities by a simple reproducible method of centrifugation of concentrated red cells in plasma at 30°C in an angle rotor at 39,000 g. The different cell fractions (vide infra) from top to bottom of the centrifugation tube (13 X 98 mm) were checked for their reticulocyte content obtained by counting 4000 cells; their red cell indices were determined with a Coulter Model S counter. For creatine measurements the separated fractions were resuspended in their original plasma and handled as outlined previously.

For the in vitro fractionation of normal blood cells, paired Student's $t$ tests were used for statistical evaluation of differences between given red cell fractions. For the in vivo studies in patients, regression equations were calculated by the least-squares method for the following relations: blood reticulocyte counts expressed in absolute numbers per ul and red cell creatine; reticulocyte counts and $T_{\text{M}}$; red cell creatine and $T_{\text{M}}$; 2,3-DPG and red cell creatine.

RESULTS

The mean creatine level determined in circulating red cells of 40 normal adults under the age of 50 yr was 5.2 ± 1.9 mg/dl of red cells (mean ± SD), which is in
Our data in 21 patients with overt hemolytic disease reveal a highly significant correlation between the number of circulating reticulocytes and the mean creatine content of the red cells \((r = 0.89, p < 0.001, \text{Fig. 1})\). Comparison was also made between the level of the oxygen-dissociation regulator 2,3-DPG and the red cell creatine content (Fig. 2) (3 patients with miscellaneous hemolytic disease and no T_{2D}Cr determinations were included in this group; in 3 of the 9 AIHA patients no 2,3-DPG levels were available). No significant correlation was found between these two variables \((r = 0.46, p > 0.05)\).

To determine the relationship between red cell creatine and reticulocytes in normally aging red cell populations, erythrocytes from 9 normal individuals were density separated according to the method described by Murphy,\(^7\) which effectively separates the cells according to age.\(^8\) The single fractions shown in Fig. 3, expressed as cumulative volumes from the top to the bottom of the centrifugation tube, demonstrated a clear relationship between the portion of reticulocytes or the density of the fraction and the creatine content: the topmost or lightest 5% of the cells showed reticulocyte enrichment of 4.7 ± 0.2% (mean ± SEM) and a creatine value of 10.9 ± 0.4 mg/dl of red cells; with progressively increasing density, the reticulocytes disappeared rapidly, and the creatine content decreased somewhat less precipitously to a value of 1.8 ± 0.3 mg/dl in the lowest or heaviest 5% of cells.

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**Fig. 1.** Relationship between number of circulating reticulocytes and mean creatine content of circulating red cells in 21 patients with hemolytic disease. Closed circles = AIHA; open circles = other hemolytic diseases.
Concomitantly, the separation method revealed the accepted characteristics of red cell aging, which means (from the top to the bottom 5% of cells) a diminution of mean cell volume from 91 ± 2 fl (mean ± SEM) to 85 ± 2 fl (p < 0.001), an increase of mean cell hemoglobin concentration from 33.8 ± 0.2 g/dl to 37.2 ± 0.4 g/dl (p < 0.001), and an unchanged mean cell hemoglobin content of 31.1 ± 0.8 pg and 31.4 ± 0.8 pg (p > 0.05), respectively. By means of a double centrifugation technique, further substantial increases in reticulocyte enrichment as well as creatine enrichment of normal blood can be obtained. The Murphy procedure was repeated with the top 13% red cell fraction pooled from eight centrifugation tubes; after the second centrifugation the top 10% red cell layer showed a reticulocyte portion of 14.3 ± 1.3% (mean ± SEM) and a corresponding creatine value of 25.5 ± 0.9 mg/dl of red cells in three such experiments.

In order to assess the predictive value of reticulocyte counts on the one hand and creatine levels on the other for rating the severity of hemolytic processes, these two parameters were correlated with red cells survival times. Graphic and statistical evaluation of these relationships indicated that a global regression analysis of all the data together reflected the real situation only insufficiently. No improvement in data representation was gained by plotting on semilogarithmic axes. The most fertile interpretation was reached by classifying the hemolytic anemias in severe
Fig. 3. Fractionation of red cells from 9 normal individuals according to density (age). The two bars on the right represent mean values (± SEM) of reticulocytes and red cell creatine in unseparated whole blood (original samples). Differences for creatine as well as for reticulocyte values between consecutive fractions were significant with \( p < 0.001 \), except for reticulocyte counts in the three middle fractions, which differed among each other with \( p < 0.01 \) (paired Student's \( t \) test).

and milder forms, setting the limit somewhat arbitrarily at 11 days \( T_{50}Cr \). Comparing the three possibilities for expressing red cell survival obtained by \( ^{51}Cr \) labeling (\( T_{50}Cr \), elution-corrected \( T_{50}Cr \), mean cell life span\(^{12} \)), the simple uncorrected \( T_{50}Cr \) was chosen because by reference to this value the most satisfactory regression coefficients were obtained (Table 2). The observation that elution correction of \( T_{50}Cr \) values gave no better correlation with creatine may be explained by the fact that both \( ^{51}Cr \) and creatine are slowly eluted red cell constituents.

The linear regression analysis between reticulocytes expressed in absolute numbers per microliter and red cell survival (\( T_{50}Cr \)) is shown in Fig. 4. In the group of severe forms of hemolytic disease (9 patients with \( T_{50}Cr \) equal to or less than 11 days) a satisfactory correlation was found between the two variables \( (r = -0.72, p < 0.05) \). In contrast, the same analysis in the group with milder hemolytic disease (13 patients with \( T_{50}Cr \) greater than 11 days) did not show a correlation \( (r = -0.28, p > 0.1) \). The graphic representation of the regression equations for the relationship between red cell creatine values and erythrocyte survival is depicted in Fig. 5. Compared to reticulocyte counts, a better correlation was found in the 9 cases with severe hemolytic disease \( (r = -0.86, p < 0.01) \). For the group with
Table 2. Correlations Between Reticulocyte Counts and Red Cell Survival and between Red Cell Creatine and Red Cell Survival in Hemolytic Diseases. Comparison Between Three Different Methods of Expressing $^{51}$Cr-Erythrocyte Survival

<table>
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<tr>
<th>Correlated Parameters</th>
<th>$T_{50}$Cr</th>
<th>$T_{50}$Cr Elution-Corrected</th>
<th>Mean Cell Life Span</th>
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<tr>
<td></td>
<td>$r$</td>
<td>$p$</td>
<td>$r$</td>
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<td>Reticulocyte counts* and red cell survival</td>
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<td></td>
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<tr>
<td>Severe</td>
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<td>&lt; 0.05</td>
<td>-0.702</td>
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<tr>
<td>Mild</td>
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<td>NS</td>
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<tr>
<td>Red cell creatine† and red cell survival</td>
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</tr>
<tr>
<td>Severe</td>
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<td>&lt; 0.01</td>
<td>-0.847</td>
</tr>
<tr>
<td>Mild</td>
<td>-0.836</td>
<td>&lt; 0.001</td>
<td>-0.721</td>
</tr>
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*Absolute numbers per microliter.
†Milligrams per deciliter of red cells.
‡Severe = 9 patients with $T_{50}$Cr $\leq$ 11 days; mild = 13 patients with $T_{50}$Cr $\geq$ 11 days.
§NS = not significant ($p > 0.05$).

milder hemolytic disease, a highly significant correlation could be detected between red cell creatine and red cell survival ($r = -0.836, p < 0.001$). It should be emphasized that by using other discriminative $T_{50}$Cr (15 days, 10 days), the pattern of results was essentially identical, with red cell creatine showing consistently closer correlations with $T_{50}$Cr than reticulocyte counts.

Fig. 4. Relationships between numbers of circulating reticulocytes and simultaneously measured red cell survival times ($T_{50}$Cr). A: Group of 13 patients with $T_{50}$Cr $\geq$ 11 days. B: Group of 9 patients with $T_{50}$Cr $\leq$ 11 days. Regression lines ± 1 SD. Closed circles = AIHA; open circles = other hemolytic diseases.
Fig. 5. Relationships between mean creatine contents of circulating red cells and simultaneously measured red cell survival times (T_{90}Cr). A: Group of 13 patients with T_{90}Cr > 11 days. B: Group of 9 patients with T_{90}Cr < 11 days. Regression lines ± 1 SD. Closed circles = AIHA; open circles = other hemolytic diseases.

DISCUSSION

Although a critical assessment of the reticulocyte count can provide an approximation of the erythropoietic activity,\textsuperscript{7,8} this cell type is too short-lived to be used as a guide to the mean age of a circulating red cell population.\textsuperscript{20,21} However, our study confirms and extends the finding that red cell creatine content is closely related to the age of these cells.\textsuperscript{11,21} The results also provide evidence that creatine measurements allow better estimation of the degree of red cell destruction than can be deduced from a so-called reticulocyte production index.\textsuperscript{21} With regard to the sensitivity of red cell creatine and reticulocyte counts to detect patients with milder hemolytic disease, we found that 3 of our 21 patients had normal creatine values (within the 95% confidence limit) despite the chromium-survival-indicated mild hemolysis (T_{90}Cr 18.1-20 days). In contrast, if one refers to absolute reticulocyte counts, 11 of the 21 patients showed normal reticulocyte counts despite mild to marked hemolysis (T_{90}Cr 13-20 days). Moreover, the variation among three to four determinations during the course of each survival study was considerably lower for red cell creatine than for reticulocyte counts (mean coefficient of variation 0.14 versus 0.29) (Table 1).

In comparison with creatine, considerable numbers of enzymes in the red cell have been used as indicators of cell age, with lowered levels associated with older cells. Of the most sensitive ones, glutamic oxaloacetic transaminase (GOT) showed three-fold to four-fold activity\textsuperscript{20,21} and hexokinase showed seven-fold to eight-fold
activity\textsuperscript{21,23,24} in extremely reticulocyte-rich red cell populations of hemolytic anemias as compared with activities found in normally aging ones. Although the latter seems promising, hexokinase is one of the least active enzymes found in erythrocytes, and its activity decreases most significantly in the 1%-2% of oldest cells only.\textsuperscript{23} Very recently, uroporphyrinogen-I-synthetase (UROS) has been proposed as another indicator of the portion of young erythrocytes in the peripheral circulation.\textsuperscript{25} The highest values reported by these investigators showed an eightfold increase over the normal mean, but most of the results were obtained in patients with sickle cell anemia, and the group of patients with other forms of considerable hemolytic disease was too small to allow a definite appraisal of the clinical value of this enzyme activity. In contrast to our results concerning the creatine level, the UROS activity correlated only roughly with reticulocyte counts.

An additional advantage of red cell creatine determinations over enzyme activity measurements is the fact that one need not worry about white cell or platelet contamination, which further reduces the technical problems of an already simple assay of a stable compound.

That the content of creatine is indeed dependent on red cell age and does not represent a variable epiphenomenon accompanying stimulated erythropoietic activity or premature bone marrow release is substantiated by our fractionation experiments of normal red cell populations. To our knowledge, there is no other erythrocytic constituent available at present that shows such large differences between old and young cells; more sophisticated cell separation techniques\textsuperscript{26} will have to be used to determine the creatine level in virtually pure reticulocyte populations.

The reason for the presence of creatine in red cells remains unknown.\textsuperscript{21} The original aim of establishing an age-dependent red cell parameter\textsuperscript{14} was temporarily obscured by a study that attempted to show an involvement of creatine in the regulation of the oxygen-carrying properties of hemoglobin.\textsuperscript{27} This study was based on an extremely heterogeneous group of patients, often with undefined anemias and involving only a small range of reticulocyte levels. In 1971 Opalinski and Beutler\textsuperscript{21} showed that creatine is chiefly related to the mean age of the red cell population and is not conditioned by the degree of anemia. The fact that we found no significant correlation between 2,3-DPG and creatine content in hemolytic diseases, together with our unpublished observations of low creatine levels in hypoplastic anemias, confirms these observations. It has been suggested that creatine is acquired by the immature red cell in its development from nuclear precursors\textsuperscript{28} and may play some part in supplying the energy requirements of protein synthesis. The possibility that red cell creatine is derived from plasma is rather improbable, since the red cell membrane offers an effective barrier to its free exchange in vitro and in vivo.\textsuperscript{28,29}

Comparison between the flatter slope of the regression equation for the relationship between red cell creatine content and T\textsubscript{50}Cr in the group of patients with severe hemolytic disease and the steeper slope representing the group of milder forms (Fig. 5) may indicate that in severe hemolysis the "shift" reticulocytes contain more creatine than do timely released red cells, although firmer binding of \textsuperscript{51}Cr by younger cells\textsuperscript{30} may produce a similar phenomenon.

Our study shows too many uncontrolled variables, and the data do not have the accuracy required to determine a mathematically defined rate of the gradual loss of
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creatine in red cells. Nevertheless, the more empirical interpretation of the results allows us to establish an effective means of classifying anemias and to obtain (under steady-state conditions) a clinically helpful and rapidly available criterion in the quantitative estimation of a process with supposedly increased red cell destruction. This information can be obtained by a simple chemical assay from a few milliliters of blood, which allows its introduction as a routine hematologic parameter.

Besides the fact that red cell creatine measurements turned out to be a sensitive indicator for the diagnosis of mild hemolytic disease (a finding that we are presently defining more precisely by correlating red cell creatine content with red cell survival times obtained from studies employing nonelutable 14C-cyanate as a red cell tag31), the introduction of creatine measurements into clinical hematology will certainly promote additional applications for this parameter. According to our experience, creatine is the best currently available index to check for steady-state conditions during any studies involving red cell kinetics. Creatine determinations allow a retrospective biopsy (similar to the use of the hemoglobin A1c level as a retrospective indicator of serum glucose elevations32) when the question of intermittent hemolysis or blood loss in the past arises but the reticulocytes have already returned to a normal level. In transfusion-requiring hemolytic anemias, repeated creatine measurements can help to determine the compatibility of the transfused red cells (prolonged lowering of creatine would indicate compatibility; quick readjustment to a patient’s pretransfusion values would reflect incompatibility, indicating an extracorpiscular hemolytic factor). In hemolytic anemias, creatine measurements of in vitro density-fractionated red cell subpopulations will help to clarify the pathogenetically important question if the destructive process is based on an age-dependent or a randomly hitting lytic mechanism. We also propose that biochemical data of erythrocytes should be more widely related to creatine contents of these cells in order to facilitate a better distinction between age- and disease-dependent alterations. This can be of practical clinical importance in situations where one has to decide about a possible deficiency of an age-dependent red cell enzyme during the phase of active hemolysis, e.g., in G-6-PD deficiency.26

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