The Removal of in Vitro Damaged Erythrocytes from the Circulation of Normal and Splenectomized Rats

By John E. Ultmann and Clara S. Gordon

The life span and sites of sequestration of erythrocytes with naturally occurring or induced red cell abnormalities in man and in animals have been studied by a number of investigators. Injuries induced by heat treatment or prolonged incubation, lecithin, arsenate, fluoride, incomplete agglutinins, metalloproteins, or by sulfhydryl inhibition with N-ethylmaleimide (NEM) or p-mercuribenzoate (PMB), have been investigated. A shortened red cell life span as well as splenic or hepatic sequestration of injured erythrocytes have been observed.

In the present study, an attempt was made to answer the following questions: (1) What is the effect of NEM treatment or prolonged incubation at 37 C. on erythrocytes and their osmotic and mechanical fragility and their filterability? (2) How do these tests compare with each other? (3) What predictability do these in vitro tests have for in vivo behavior of erythrocytes? (4) What is the role of spleen, liver, and bone marrow in the process of sequestration? (5) What is the effect of splenectomy on erythrocyte survival and on the patterns of sequestration?

Materials and Methods

Bartonella-free, white, male, Sprague-Dawley rats weighing between 350 and 450 Gm. were housed 3 to a cage and fed Rockland Laboratory animal diet and water ad libitum. Splenectomy was performed under ether anesthesia through a midline incision at least 4 weeks prior to the life span studies. Each experimental group consisted of 6 rats.

Sodium Chromate Labeling

Heparinized blood was obtained from the inferior vena cava of pentothal-anesthetized rats. The whole blood was incubated with 10 μc Na₂Cr⁵¹O₄ (218–393 mc./mg.; Chromotope sodium, Squibb®) per ml. at room temperature for 30 minutes. Labeling was terminated by the addition of 10 mg. sodium ascorbate per ml. of blood and after 2 minutes the erythrocytes were washed two times with three volumes of 0.85 per cent saline. Total radioactivity administered to each animal was calculated from a 0.1 ml. aliquot of labeled blood.

NEM Treatment

Following Cr⁵¹-labeling and washing, the erythrocytes were incubated for 2 hours at 37 C. with NEM. To a 50 per cent red cell suspension in isotonic phosphate buffer (0.1

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M, pH 7.4, 7 mEq./L K+, and 150 mEq./L Na+) was added an equal volume of sulfhydryl inhibitor in saline to obtain a final concentration of 5 to 40 μM N-ethylmaleimide (NEM; Schwarz BioResearch, Inc., Orangeburg, New York) per ml. packed red blood cells. Dextrose was added to provide a final concentration of 200 mg. per 100 ml.10,11

Control blood samples were incubated in buffer-dextrose-saline, but without NEM, for 2 hours at room temperature.

**Incubation in Water Bath at 37 C**

Defibrinated blood was incubated in a thermostatic water bath at 37 C. for 21 hours. The blood was subsequently labeled with chromium51 (see above) and is referred to in the text as "prolonged incubation."

**Preparation of Labeled Hemoglobin**

Cr51-labeled red cells were lysed by osmotic shock.12 Stroma, leukocytes, and thrombocytes were removed by centrifugation at 4000 g. for 45 minutes, and the clear supernatant fluid was used.

**Osmotic Fragility**

This was determined by a modified Cr51 differential osmotic fragility technic.13,14 Hemolysis was expressed as per cent radioactivity found at each NaCl concentration as compared to total hemolysis. Osmotic fragility curves and their derivatives were also determined by Danon's method using a "Fragiligraph" for automatic recording.15

**Mechanical Fragility**

This was measured by the method of Shen et al.16 adapted for isotopic determination.13,14 The following formula was used: Mechanical Fragility = \( \frac{[C - A] \times 100}{[B - A]} \),

where \( A \) = cpm in supernatant of blood placed in 1.25 per cent NaCl, as undamaged control; \( B \) = cpm in supernatant of blood placed in distilled water; and \( C \) = cpm in supernatant of blood subjected to standard mechanical trauma.

**Millipore Filterability**

Erythrocytes were passed through millipore filters under low pressure, employing a modification of the technic described by Jandl17 adapted for isotopic determination.13,14 The degree of hemolysis was determined following the passage of the cells through a series of ten 10-micron millipore filters (OSWPO 1300, Millipore Filter Corp., Bedford, Mass.) under a pressure of 226.1 Gm./cm.2 The same formula as for mechanical fragility was then used. In this test, the degree of hemolysis is a measure of the loss of reversible deformability (plasticity) of the erythrocytes; that is, the cells, which have passed through the filters have been deformed and have failed to withstand the stress of temporary alteration of shape, are hemolyzed.

**Reinjection of Labeled Blood**

Half a ml. of Cr51-labeled erythrocytes was reinjected into the tail vein.

**Determination of Erythrocyte Clearance**

At 10, 30, 60, 120, 180, and 240 minutes, 0.02 ml. of blood was removed with certified hemoglobin pipettes from the cut tail tip of each rat. The radioactivity of the blood was determined in a well-type scintillation counter; sufficient counts were obtained to reduce sampling error to 2 per cent or less. Counts were corrected for background (130-160/ min.). To calculate the red cell removal rate, the results were plotted on semilogarithmic paper and the 100 per cent level estimated by extrapolation to zero time. The erythrocyte
REMOVAL OF IN VITRO DAMAGED ERYTHROCYTES

survival in the circulation was then replotted as per cent of radioactivity remaining at various time intervals as compared to the estimated 100 per cent level at zero time.

Determination of Organ Radioactivity
All rats were sacrificed 4 hours after injection of labeled red blood cells. Spleen, liver, kidneys, lungs, and heart were removed, weighed, and the radioactivity was determined on samples of tissue in the well-type scintillation counter. Muscle and connective tissue were stripped off bones (scapulae, humeri, radii, ulnae, vertebrae, sacrum, pelves, femora, tibiae, and fibulae) and “bone marrow” radioactivity was determined. Total radioactivity was calculated for all organs and the results expressed as per cent of calculated radioactivity administered. Specific radioactivity could be determined for the organs but not for “bone marrow.” Standard deviation and “p” values were calculated according to Snedecor.18

Plasma Hemoglobin Clearance and Sites of Removal
At 2, 5, 8, 10, 30, 60, 120, 180, and 240 minutes following injection of 0.7 ml. labeled hemoglobin, 0.02 ml. of blood was removed from the cut tail tip of each rat and its radioactivity determined. Clearance rate was calculated as outlined above for erythrocytes. The organ distribution of removed, labeled hemoglobin was determined as described above.

RESULTS

A. In Vitro Experiments

1. Osmotic fragility. Figures 1A and 1B depict the osmotic fragility of normal and injured erythrocytes. Following treatment with 5 or 10 \( \mu \text{M} \) NEM per ml. packed red blood cells (\( \mu \text{M NEM/ml. RBC} \)), there was no consistently detectable alteration in osmotic fragility, whereas with 20 \( \mu \text{M NEM/ml. RBC} \) a significant portion of red blood cells showed decreased osmotic fragility. Larger doses of NEM caused a progressive increase in fragility of the majority of the red cells. Twenty-one-hour incubation at 37 C. resulted in an intermediate increase in fragility. The derivative curve obtained from the “Fragiligraph” (fig. 1C) indicates the population distribution according to osmotic fragility of erythrocytes altered by NEM or by prolonged incubation as compared to the symmetric distribution of normal red blood cells.

2. Mechanical fragility. Mechanical fragility of normal and injured erythrocytes is compared in table 1. Red blood cells treated with more than 10 \( \mu \text{M} \) NEM/ml. RBC or by prolonged incubation at 37 C. exhibited an increase in mechanical fragility compared to normal.

3. Millipore filterability. Millipore filterability of normal and damaged erythrocytes is compared in table 1. Exposure of erythrocytes to 5 and 10 \( \mu \text{M} \) NEM/ml. RBC resulted in no detectable alteration of millipore filterability. Treatment with 20 \( \mu \text{M} \) NEM/ml. RBC produced cells with some increase in plasticity capable of passage through the filters without destruction. With increasing chemical injury or following prolonged incubation, the plasticity of the erythrocytes decreased and more cells were hemolyzed during passage through the filters than in the control study.

B. In Vivo Experiments

1. RBC survival and sites of sequestration of normal erythrocytes. Ten to 30 minutes following the injection of untreated red cells into normal or
spleenectomized animals, the radioactivity of the blood stabilized at about 90 per cent of initial values and did not change for the remainder of the experiment (fig. 2A). The mean radioactivity of the organs examined was less than 5 per cent (fig. 3A).

2. Effect of two levels of NEM on RBC survival and sites of sequestration.
   a. Twenty $\mu$M NEM/ml. RBC. Injection of these cells into normal animals resulted in their clearance from the blood in an exponential fashion with a mean half-clearance time of 78 minutes (fig. 2B) mainly by splenic sequestration (fig. 3B). Mean total splenic radioactivity was 57 times greater than in control animals ($p < 0.01$). Mean total liver radioactivity doubled ($p < 0.05$), whereas "bone marrow" activity increased two and one-half times ($p < 0.05$) compared to the normal controls. Total radioactivity of spleen was five times greater than liver and eight times greater than "bone marrow"
REMOVAL OF IN VITRO DAMAGED ERYTHROCYTES

Table 1.—Mechanical Fragility and Millipore Filterability of Normal and Injured Rat Erythrocytes

<table>
<thead>
<tr>
<th>Erythrocytes Studied</th>
<th>Mechanical Fragility Per Cent*</th>
<th>Millipore Filterability Per Cent*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>6.4 (5)†</td>
<td>2.1 (12)†</td>
</tr>
<tr>
<td>5 μM NEM/ml.</td>
<td>2.4 (3)</td>
<td>2.5 (2)</td>
</tr>
<tr>
<td>10 μM NEM/ml.</td>
<td>7.3 (3)</td>
<td>2.7 (2)</td>
</tr>
<tr>
<td>20 μM NEM/ml.</td>
<td>13.9 (5)</td>
<td>0.8 (8)</td>
</tr>
<tr>
<td>21 hrs. incubation at 37 C.</td>
<td>16.6 (2)</td>
<td>7.3 (3)</td>
</tr>
<tr>
<td>30 μM NEM/ml.</td>
<td>25.0 (3)</td>
<td>28.0 (3)</td>
</tr>
<tr>
<td>35 μM NEM/ml.</td>
<td>27.4 (1)</td>
<td></td>
</tr>
<tr>
<td>40 μM NEM/ml.</td>
<td>43.8 (2)</td>
<td>52.6 (3)</td>
</tr>
</tbody>
</table>

*% = \( \frac{(C - A) \times 10^2}{B - A} \), where A = cpm in supernatant of blood placed in 1.25 per cent NaCl as undamaged control; B = cpm in supernatant of blood placed in distilled water (representing total hemolysis); and C = cpm in supernatant of blood subjected to standard mechanical trauma or to passage through a series of ten (10-micron) millipore filters.

†Number of experiments averaged.

with the NEM-treated blood. Specific radioactivity of spleen was 66 times greater than that of liver in the case of the 20 μM NEM-treated erythrocytes, whereas it was only three times greater with normal cells (table 2).

b. Forty μM NEM/ml. RBC. These erythrocytes were cleared from the circulation with a half-time of 35 minutes (fig. 2C), but, in contrast to the less damaged cells, splenic sequestration did not play an important role. Mean total splenic radioactivity was less than 10 per cent \( (p < 0.01) \) of the 20 μM NEM-treated cells (fig. 3C). Mean total liver radioactivity was two times greater \( (p = 0.01) \) than with the 20 μM NEM-treated cells and five times greater \( (p = 0.01) \) than with the normal erythrocytes. In contrast to the less damaged cells, total liver radioactivity was more than four times total splenic activity. Specific activity of the spleen was only three times greater than that of liver (table 2). "Bone marrow" total radioactivity was essentially the same as with the control blood. With the 40 μM NEM-damaged cells, total kidney radioactivity was six and ten times greater \( (p = 0.05) \) than with normal or 20 μM NEM-damaged erythrocytes, respectively.

c. Effect of splenectomy. Following splenectomy, the life span of the 20 μM NEM/ml. RBC-damaged erythrocytes was significantly increased. After 240 minutes (fig. 2B), 67 per cent of the injected radioactivity remained in circulation, resulting in an estimated half-clearance time of over 500 minutes. The mean total radioactivity of the liver doubled \( (p = 0.05) \) following splenectomy, whereas the other organs showed no significant change (fig. 3B); however, only 33 per cent of the erythrocytes had been cleared when the experiment was terminated.

With the 40 μM NEM/ml. RBC-damaged erythrocytes, no significant change in red cell survival time was observed following splenectomy (fig. 2C). The mean total radioactivity of the liver increased 1.5 times, which was not considered significant \( (p = 0.1) \). The radioactivity of other organs also did not change (fig. 3C).
3. **Effect of prolonged incubation at 37 C. on RBC survival and sites of sequestration.** The erythrocytes were cleared from the circulation with a half-time of 50.4 minutes in normal rats and in 59.0 minutes in splenectomized animals, the difference not being significant. The mean total radioactivity of the spleen was equal to the total radioactivity of the liver (fig. 4), whereas the specific activity of the spleen was 12 times greater than that of the liver (table 2). The other organs did not show any significant increase in radioactivity as compared to normal controls. Following splenectomy, mean total radioactivity of the liver nearly doubled ($p = 0.02$), whereas other organs showed no significant change.

4. **Clearance and sites of removal of labeled hemoglobin.** Labeled hemoglobin was injected into a group of rats and the disappearance rate and sites of accumulation of the hemoglobin were determined. After 57 minutes, 50 per cent of the radioactivity remained in circulation (fig. 5). The organ distribution of Hb-Cr\textsuperscript{51} at the end of 240 minutes is shown in figure 6. It is apparent that in contrast to the 40 $\mu$M/ml RBC-injured erythrocytes, the clearance rate is slower, and at the end of 240 minutes, there is less radioactivity in the liver and a greater amount of radioactivity in the kidneys.

**DISCUSSION**

*Effect of Chemical Injury or Prolonged Incubation of Erythrocytes on in Vitro Tests*

The osmotic fragility test is a measure of erythrocyte shape and size reflecting the capacity of these cells to withstand osmotic lysis. Whereas levels
Fig. 3.—Organ distribution of Cr⁴⁺-labeled erythrocytes.

of 5 and 10 μM NEM/ml. RBC have negligible effect on the red cells, the 20 μM NEM/ml. RBC dose produces erythrocytes more resistant to osmotic lysis than normal cells. With the 30 and 40 μM NEM/ml. RBC levels, the capacity to withstand osmotic lysis decreases. Similar results have been reported by Jacob and Jandl¹⁰ who showed, in addition, that 20 μM NEM-treated cells were smaller than normal. The 21-hour incubation of red cells at 37 C. was specifically selected to produce an alteration which would result in osmotic fragility characteristics approximating those achieved by chemical means with 25 to 30 μM NEM/ml. RBC incubation, that is, midway between the osmotic fragility of normal and 40 μM NEM-injured red cells.

The mechanical fragility test may be considered a measure of the strength
Table 2.—Mean Specific Cr\textsuperscript{31}-Activity of Organs\textsuperscript{*}

<table>
<thead>
<tr>
<th>Erythrocytes Studied</th>
<th>Spleen</th>
<th>Liver</th>
<th>Kidneys</th>
<th>Lungs</th>
<th>Heart</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Normal</td>
<td>Splx</td>
<td>Normal</td>
<td>Splx</td>
<td>Normal</td>
</tr>
<tr>
<td>Untreated RBC</td>
<td>1726</td>
<td>—</td>
<td>525</td>
<td>528</td>
<td>516</td>
</tr>
<tr>
<td>20 (\mu)M NEM/ml. RBC</td>
<td>75,657</td>
<td>—</td>
<td>1147</td>
<td>2143</td>
<td>324</td>
</tr>
<tr>
<td>RBC incubated</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>21 hrs. at 37 C.</td>
<td>19.814</td>
<td>—</td>
<td>1671</td>
<td>2923</td>
<td>921</td>
</tr>
<tr>
<td>40 (\mu)M NEM/ml. RBC</td>
<td>8936</td>
<td>—</td>
<td>2589</td>
<td>3533</td>
<td>3318</td>
</tr>
</tbody>
</table>

\textsuperscript{*}Mean specific Cr\textsuperscript{31}-activity of organs (cpm/Gm. tissue; 6 rats in each group).
\textsuperscript{†}Normal—normal rats.
\textsuperscript{†}Splx—splenectomized rats.
of the red cell membrane; degree of spheroidicity and agglutinability may also play a role. With increasing amounts of NEM, there is a progressive increase in mechanical fragility. The 10 and 20 μM NEM/ml RBC levels, which have no effect on or decrease the osmotic fragility, respectively, produce significant increases in mechanical fragility. The erythrocytes subjected to prolonged incubation have mechanical fragility characteristics of a moderately injured cell. The 40 μM NEM-injured erythrocytes show a marked increase in mechanical fragility.

The millipore filterability test is thought to be a measure of the reversible deformability (plasticity) of erythrocytes. Under conditions of the present experiment, using a series of filters, erythrocytes passing through the pores of the filters are deformed and hemolyzed if unable to withstand the alteration in shape. Following treatment with 20 μM NEM/ml RBC, fewer cells are hemolyzed than with normal blood. This finding may reflect, in part, an increase in plasticity, and, in part, the decrease in cell size. With more profound injury there is a decrease in plasticity.

From the present study, we conclude that chemical alteration or prolonged incubation of erythrocytes leads to profound changes in in vitro tests of red cell integrity. With moderate or severe injury, the results in the three test systems studied—osmotic and mechanical fragility and millipore filterability—correlate roughly with the degree of injury applied. With the mild injury, however, only the mechanical fragility predicts the in vivo events.

Effect of Chemical Alteration or Prolonged Incubation of Erythrocytes on Their Life Span and Sites of Sequestration

The 20 μM NEM/ml RBC-injured red cells are cleared from the circulation with a half-time of 78 minutes, a rate comparable to that reported by others. It has been calculated that this rate is limited largely by the rate
The present study further emphasizes the role of the spleen in clearance of mildly injured cells. There is accumulation of radioactivity predominantly in the spleen, and following splenectomy the clearance time increases markedly. Because of the degree of damage produced with 20 μM NEM, splenectomy does not correct the life span to normal values and the liver becomes responsible for the major portion of continued sequestration. The bone marrow does not show an increase in sequestration following splenectomy. The postsplenectomy data in our study are an approximation of the organ distribution and are not completely comparable to the presplenectomy values as only 33 per cent of the erythrocytes had been cleared when the experiment was terminated. The error in these data is in the direction of underestimating the pre- and postsplenectomy differences.

The injury produced by incubation at 37 C. for 21 hours was chosen to provide moderately injured cells. Their clearance is more rapid than that of cells treated with the 20 μM NEM/ml. RBC, and splenic sequestration is approximately equal to hepatic sequestration. The beneficial effect of splenectomy on erythrocyte survival is minimal. The hepatic reticuloendothelial elements appear capable of clearing these cells almost as effectively as the
REMOVAL OF IN VITRO DAMAGED ERYTHROCYTES

The most injured erythrocytes (40 μM NEM/ml. RBC) are markedly altered in all three in vitro tests. In some of the experiments, a minimal amount of hemolysis occurred in isotonic saline. Erythrocyte clearance is very rapid. Almost all of the radioactivity is taken up by the liver. Sequestration by the spleen and bone marrow appears to be of minor significance. The effect of splenectomy is negligible. Radioactivity is accumulated in the kidneys, both pre- and postsplenectomy, suggesting that some degree of intravascular hemolysis has taken place. Injected hemoglobin, however, is removed from the circulation at a slower rate than the severely injured red cells, and more radioactivity is found in the kidneys than in the liver, findings different from those noted with the 40 μM NEM-injured erythrocytes. Similar observations have been reported by Wagner et al. in man. It is unlikely that the markedly injured cells might have been sequestered by the spleen and the radioactivity released from there and taken up by the liver, since it has been shown in man that Cr51 of labeled erythrocytes trapped in spleen is released at a rate approximating 4 per cent per day.7

The effect of NEM treatment or of thermal injury on erythrocyte metabolism and life span has been studied by a number of investigators. Jacob and Jandl have pointed out that the freely permeable sulfhydryl inhibitor, N-ethylmaleimide (NEM), causes a fall in intracellular glutathione, inhibition of glucose consumption and lactate formation, loss of cellular potassium, and gain of sodium. The influx of water leads to sphering, swelling, and eventual hemolysis. Dawson and Widdas have recently reported that incubation of red cells with NEM produces inhibition of the facilitated trans-

Fig. 6.—Sites of removal of Cr51-labeled hemoglobin.
fer of glucose. Wagner et al. noted that heat treatment produced no significant change in glutathione, whereas there was marked reduction in glucose-6-phosphate dehydrogenase. In addition to spherling, formation of "tear drop" cells was noted. NEM-treated or thermally injured erythrocytes undergo biochemical and biophysical changes similar but not entirely identical to those resulting from in vivo aging. The critical biochemical or biophysical defect leading to red cell sequestration and destruction of injured or aged cells remains unknown.

Jacob and Jandl, Wagner, et al., and Crome and Mollison observed distinctive patterns of alterations of erythrocyte life span and subsequent sequestration depending more on the degree of damage than on its type. These patterns correlated roughly with alterations in osmotic fragility observed in vitro. Mollison and his associates and Jandl and Kaplan have studied the effects of progressively increasing quantities of anti-B and anti-D antibodies on B and D cells, respectively. They found that the sites of erythrocyte trapping were determined by quantitative factors: the spleen removes from the circulation red cells the surface properties of which are so slightly changed as to pass through the liver intact. Jacob and Jandl have pointed out that the specificity with which the spleen traps sulfhydryl-deficient cells is striking and may even exceed that of red cells with anti-D antibody. The means by which the reticuloendothelial system, particularly the spleen, specifically recognizes erythrocytes altered by chemical, physical, or immune injury, or by age, is not yet clear.

**Summary**

The in vitro alterations and in vivo fate of erythrocytes treated with N-ethylmaleimide or subjected to prolonged incubation were studied in normal and splenectomized rats. Minimal injury (20 μM NEM/ml RBC) resulted in red cells with decreased osmotic fragility and increased plasticity; however, mechanical fragility was significantly increased. These cells were removed with a half-time of 78 minutes, mainly by splenic sequestration, and splenectomy prolonged their life span. Incubation at 37 C. for 21 hours produced erythrocytes with increased osmotic and mechanical fragility and decreased plasticity. Erythrocyte clearance was more rapid (T½ = 59 minutes), with spleen and liver removing approximately an equal number of cells and splenectomy having little effect on red cell life span. With severe injury (40 μM NEM/ml RBC), all three in vitro measurements showed marked alterations, red cell removal was rapid (T½ = 35 minutes), mainly by hepatic sequestration, and clearance was unaffected by splenectomy.

The present studies have shown that chemical injury or prolonged incubation lead to profound changes in in vitro tests of red cell integrity, the mechanical fragility predicting most closely the subsequent in vivo events. Although the entire reticuloendothelial system appears to participate in red cell removal, the spleen and liver are the major sites of sequestration in the rat. The splenic removal predominates with minimally injured cells, hepatic
removal with moderately and severely altered cells. The type of injury appears to be of less significance than the degree of injury of the red cells.

**SUMMARIO IN INTERLINGUA**

Le alterationes in vitro e destino in vivo de erythrocytos tractate con N-ethylmaleimida o subjicite a un prolongate incubation esseva studiate in rattos normal e in rattos splenectomisate. Un lesionage minimal (20 \( \mu \)M de n-ethylmaleimida per ml de erythrocytos) resultava in cellulas con reducute fragilitate osmotic e augmentate plasticitate. In plus, le fragilitate mechanic del cellulas esseva augmentate a grados statisticamente significative. In vivo iste lesionate erythrocytos esseva eliminate con 78 minutas como tempore de medie valor. Le mechanismo del elimination esseva primarimente sequestration splenic, e splenectomia effectuava un prolongate durate vital del cellulas. Vinti-un horas de incubation a 37 C produceva erythrocytos con augmentate fragilitate osmotic e mechanic e reducute plasticitate. In iste situation le elimination del erythrocytos esseva plus rapide. Le tempore de medie valor esseva 59 minutas. Sequestration splenic e sequestration hepatic esseva responsabile pro approximativemente equal numeros de cellulas eliminate. Splenotomia habeva pauc effecto super le durata vital del cellulas. Post lesionage sever (20 \( \mu \)M de N-ethylmaleimida per ml de erythrocytos) omne le tres mentionate parametros monstrava marcate alterationes in vitro. Le elimination del cellulas ab le circulation esseva plus rapide. Le valor de medie tempore esseva 35 minutas. Le mechanismo del elimination esseva primarimente sequestration hepatic. Splenotomia exerceva nulle effecto super le processo.

Le presente studios ha demonstrate que le lesionage chimic o le prolongate incubation de erythrocytos resulta in profunde alterationes del comportamento del cellulas in tests in vitro. Le mechanic fragilitate in vitro predice le plus proximemente le subsequente evenimentos in vivo. Ben que le total systema reticuloendothelial pare participar in le elimination del erythrocytos, le splen e le hepate es le major sitos del sequestration in le ratto. Sequestration splenic predomina in le caso de minimalmente lesionate cellulas, sequestration hepatic in le caso de cellulas moderamente o severmente alterate. Le typo de lesionage del erythrocytos pare esser minus significative que le grado de illo.

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**REFERENCES**

11. —, and —: Effects of sulphydryl inhibition on red blood cells. II. Stud-
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