Surface Remodeling vs. Whole-Cell Hemolysis of Reticulocytes Produced With Erythroid Stimulation or Iron Deficiency Anemia

By Steven E. Come, Stephen B. Shohet, and Stephen H. Robinson

32P in membrane phosphatidylethanolamine (PE) and red cell 14C, reflecting cytoplasmic hemoglobin, were measured sequentially in rats given transfusions of doubly-labeled reticulocytes. With reticulocytes from normal rats there was a small decline in the levels of both the membrane and the cytoplasmic labels; the changes were almost parallel, although loss of membrane PE-32P exceeded that of 14C to a small extent. By contrast, with “stress reticulocytes” from bled donors, there was a markedly disproportionate loss of the membrane label; this asymmetrical loss of membrane material was diminished when recipients had been splenectomized. With transfusions of doubly-labeled reticulocytes from rats with severe iron deficiency anemia, there was a marked loss of both membrane PE-32P and red cell 14C which was only moderately asymmetrical.

The asymmetrical loss of the membrane label found with stress reticulocytes supports the conclusion that these cells undergo a process of surface remodeling during their maturation in the peripheral blood. The spleen is partly responsible for this process. Normal reticulocytes also appear to undergo a minor degree of remodeling. On the other hand, the almost symmetrical loss of membrane and cytoplasmic label observed with reticulocytes from iron deficient rats indicates that many of the cells in this model of ineffective erythropoiesis are hemolyzed in their entirety. These experiments demonstrate that stress reticulocytes differ under different conditions and may lose cellular material by two, possibly interrelated, mechanisms: surface remodeling or whole-cell hemolysis.

Reticulocytes produced in response to erythroid stimulation are unusually large.2-5 Isotopic studies have suggested that these “stress reticulocytes” have a shortened life-span in the circulation.4-10 This conclusion appeared to be confirmed by recent experiments showing that degradation of hemoglobin from these cells accounts in large part for the enlarged erythropoietic fraction of bilirubin found with increased rates of red cell production.11 On the other hand, Ganzoni and co-workers have presented evidence that stress reticulocytes are not hemolyzed but undergo a process of surface remodeling during which only portions of their cytoplasm are lost.12

Presented in part at the annual meeting of the American Society for Clinical Investigation, May 1971.

1974 by Grune & Stratton, Inc.
laboratory have supported the latter concept, as have recent studies by Shattil and Cooper. The present investigation was undertaken to extend our preliminary findings, to examine the role of the spleen in this process, and to investigate the fate of reticulocytes from rats with severe iron-deficiency anemia. This condition in rats is associated with marked ineffective erythropoiesis and with a striking loss of reticulocyte hemoglobin. It was therefore of interest to compare the fate of reticulocytes in this model of pathologic ineffective erythropoiesis with that observed with physiologically regulated erythroid hyperplasia in normal rats responding to blood loss.

The present experiments were predicated on the feasibility of following the fate of stable, discrete radioactive labels in the cell membrane and the cytoplasm of maturing reticulocytes. These criteria are well fulfilled with the use of inorganic $^{32}$P to label the membrane lipid phosphatidylethanolamine (PE) and glycine-2-$^{14}$C to label cytoplasmic hemoglobin. The only erythroid cells that can be labeled in this fashion are immature forms that are still synthesizing cell membrane and hemoglobin, and these cells remain so labeled throughout their life span. If such doubly-labeled cells are destroyed in their entirety, there should be symmetrical loss of both the membrane and the cytoplasmic labels. With surface remodeling, on the other hand, one might expect a disproportionate decrease in the membrane label because of the loss of surface-rich portions of the cell with concomitant resealing of the cell membrane.

**MATERIALS AND METHODS**

Cesarian-delivered 250-400 g male Sprague-Dawley rats from a Bartonella-free colony (Charles River Farms, Wilmington, Mass.) were used throughout these experiments.

**Techniques of Labeling Cell Membrane and Cytoplasm**

Doubly-labeled reticulocytes were obtained from normal, bled, or iron-deficient donor rats by the simultaneous intravenous injection of 50 µCi glycine-2-$^{14}$C (18 mCi/mM, New England Nuclear Corp.) to label cytoplasmic hemoglobin and 3-5 mCi $^{32}$P (approximately 300 mCi/mM carrierfree neutralized phosphoric acid, New England Nuclear Corp.) to label membrane PE. Twenty-four hours later, the donor rats were exsanguinated by cardiac puncture. After excess plasma had been removed, the cells were resuspended in isotonic saline and volumes containing 0.3-0.6 g hemoglobin were used for transfusion. This was equivalent, in ml of red cells, to 7% of the recipients' red cell volumes; similar volumes were used for all experimental groups and the findings in each group did not vary in relation to the amount of blood transfused. Aliquots were retained for reticulocyte counts and determination of routine hematologic values with a Coulter model S counter.

Recipients rats were transfused under ether anesthesia by slow injection through a tail vein. Blood samples of 1.5-2.0 ml were collected into 0.5 ml ACD solution from a tail vein at 0.5, 4, 24, 48, and 72 hr following transfusion. These samples and a portion of the blood used for transfusion were washed three times with isotonic saline. Theuffy coat was removed after each wash. It is possible that some reticulocytes may have been removed along with theuffy coat; however, the findings for reticulocyte labeling were highly consistent from experiment to experiment. It is also possible that some labeled white cells were not eliminated with the washes, but trial experiments in which cells from theuffy coat were added back to washed red cell suspensions indicated that at least 95% of the leukocytes had in fact been removed. The packed red cells were resuspended in saline, aliquots were removed for measurement of hematologic values, and the remainder was used for determination of $^{14}$C and PE-$^{32}$P specific activity. It was assumed that reutilization of these labels in the recipients was negligible because of the initial hypertransfusion and the brief period of study thereafter.
Analysis of Radioactivity

Total $^{14}$C radioactivity was assayed by applying 20 $\mu$l of the final red cell suspension to 15-mm diameter filter papers which were immersed in 10 ml Buhler's solution and counted in a liquid scintillation spectrometer.

Red cell ghosts were prepared from measured volumes of the cell suspension according to the method of Dodge and Hanahan with the exception that the hypotonic media were added to the cells at room temperature rather than at 4°C. Total lipids were then extracted from the ghost preparations. These methods have been shown to permit virtually complete recovery both of ghosts from red cell suspensions and of lipids from ghost preparations. PE-$^{32}$P was isolated from the total lipid by thin layer chromatography using a minor modification of Skipski's method. The PE band was eluted, the PE concentration measured, and $^{32}$P radioactivity determined with a liquid scintillation spectrometer.

To obtain an expression of the relationship between the changes in membrane and cytoplasmic labeling as a function of time, the ratio of PE-$^{32}$P to $^{14}$C was calculated for each sample assayed. The ratio of these values in the blood used for transfusion was adjusted to one and the subsequent ratios found in animals receiving this blood were calculated in terms of this “zero time” value.

Preparation of Stress, Normal, and Iron-deficient Reticulocytes.

Stress reticulocytes were produced by removing 35% of the blood volume of the donor rats both 3 days and 1 day before injection of glycine-$^{14}$C and $^{32}$P. The labeled blood, with a mean hemoglobin concentration of 8.1 g/100 ml, mean corpuscular volume (MCV) 66.0 cu $\mu$, and reticulocyte count 22%, was harvested 24 hr later. Littermates not bled previously were exsanguinated 24 hr after administration of the same isotopes to yield normal reticulocytes (mean hemoglobin concentration 14.4 g/100 ml, MCV 58.0 cu $\mu$, reticulocyte count 1.6%). Iron-deficient donors were rendered iron deficient from the time of weaning by special diet. Twelve such donors, approximately 6-8 wk old and weighing 80-100 g, received similar isotope injections and were exsanguinated 24 hr later. The mean hemoglobin concentration of this blood was 2.9 g/100 ml and the MCV 31.2 cu $\mu$. As found previously, the iron deficient rats manifested moderate reticulocytosis, averaging 8.7%.

Validation of Experimental Design.

Stability of PE-$^{32}$P as a membrane label. Two in vitro experiments were performed. In the first study donor rats were bled according to the schedule used to obtain stress reticulocytes and were then given injections of $^{32}$P alone. Twenty-four hours later blood was harvested under sterile conditions. The cells were washed once with normal rat serum, and 0.3 ml samples were incubated sterilely with 1.5 ml normal rat serum including 4 mg glucose, 200 $\mu$g penicillin, and 200 $\mu$g streptomycin at 37°C with gentle agitation in a Dubnoff metabolic shaker. The pH was maintained between 7.1-7.7 and an additional 1 mg glucose was added after 12 hr of incubation. Hematologic values and PE-$^{32}$P labeling of red cell ghosts were determined from duplicate samples taken over 32 hr of incubation.

The second experiment was similar to the first except that no isotope was given to the donor rats. Instead, 0.1 mCi $^{32}$P was added to the serum in which the unlabeled erythrocytes were incubated. PE-$^{32}$P labeling was determined in ghost preparations of cells harvested at the same intervals as in the first experiment.

Morphology of cell membrane preparations. Ghosts derived from stress reticulocytes were evaluated by both electron microscopy and histochemical techniques. For electron microscopy whole red cells or ghosts were prepared by previously described techniques. Intact stress reticulocytes and ghosts prepared from these cells were also stained with methyl green pyronin, Janus green, and phosphotungstic acid-hematoxylin to confirm the absence of significant quantities of microsomes or mitochondria from these preparations.

Validity of total $^{14}$C counts as a reflection of hemoglobin label. Four rats received transfusions of labeled stress reticulocytes prepared as described above except that glycine-$^{14}$C alone was given to the donor animals. Samples were taken at the usual times for measurement of the specific activities of both total red cell $^{14}$C and hemoglobin hemin-$^{14}$C crystallized from these cells.
For comparison with total $^{14}$C dpm/10$^8$ RBC, dpm/mg hemin was converted to dpm in hemin/
10$^8$ RBC on the basis of the molecular weights of hemin and hemoglobin and the mean corpuscular
hemoglobin of the blood sample.

**Effects of osmotic hemolysis of whole cells.** Two normal rats were transfused with doubly
labeled normal reticulocytes obtained from unbled donor rats. Blood samples were drawn 0.5 and
4 hr after transfusion for assay of red cell $^{14}$C and membrane PE-32P. A polyethylene
catheter was then secured in the tail vein, and distilled water was infused at a rate of 1–4 ml/hr
over the next 44 hr. Additional blood samples were drawn at the termination of the water in-
fusion and again 24 hr later.

**RESULTS**

**Experimental Findings for Normal and Stress Reticulocytes**

In the experiments with labeled normal reticulocytes, there was a moderate
loss of both membrane PE-32P and total red cell $^{14}$C (Table 1, Fig. 1). 32P-PE fell
to a slightly greater extent than $^{14}$C; the difference in these two curves was statistically
significant with $p = 0.005$ by the Wilcoxin matched-pairs signed-ranks test.$^{33}$ However, the loss of the two labels was almost parallel, the final ratio of
PE-32P/$^{14}$C ranging from 0.91 to 0.97 as compared to 1.0 in the transfused cells
(Fig. 2).

The findings with stress reticulocytes from bled donors were in sharp contrast
to those for normal cells. Again there was a moderate fall in $^{14}$C labeling, but
now a striking decline in 32P radioactivity (Table 1, Fig. 1). This dis propor-
tionate loss of the membrane label is reflected by the final ratio of PE-32P/$^{14}$C
which ranged from 0.32 to 0.38 as compared to 1.0 in the transfused cells
(Fig. 2).

When labeled normal reticulocytes were transfused into recipients that had
been splenectomized 1 wk earlier, PE-32P, $^{14}$C, and the PE-32P/$^{14}$C ratio all de-

---

**Table 1. Changes in Membrane PE-32P and Total RBC-$^{14}$C in
Rats Transfused With Doubly-Labeled Reticulocytes**

<table>
<thead>
<tr>
<th>Donor Cells</th>
<th>Recipients</th>
<th>0.5 hr</th>
<th>72 hr</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PE-32P</td>
<td>$^{14}$C</td>
<td>PE-32P</td>
</tr>
<tr>
<td></td>
<td>dpm/10$^8$ RBC (%)</td>
<td>dpm/10$^8$ RBC (%)</td>
<td>dpm/10$^8$ RBC (%)</td>
</tr>
<tr>
<td>Normal reticulocytes</td>
<td>4</td>
<td>482 ± 19</td>
<td>100</td>
</tr>
<tr>
<td>Normal reticulocytes (Splenx)</td>
<td>2</td>
<td>476 ± 19</td>
<td>100</td>
</tr>
<tr>
<td>Normal reticulocytes (Distilled water hemolysis)</td>
<td>2</td>
<td>621 ± 19</td>
<td>100</td>
</tr>
<tr>
<td>Stress reticulocytes</td>
<td>4</td>
<td>4745 ± 236</td>
<td>100</td>
</tr>
<tr>
<td>Stress reticulocytes (Splenx)</td>
<td>2</td>
<td>5180 ± 236</td>
<td>100</td>
</tr>
<tr>
<td>Iron- deficient reticulocytes</td>
<td>4</td>
<td>5250 ± 236</td>
<td>100</td>
</tr>
<tr>
<td>Iron- deficient reticulocytes</td>
<td>4</td>
<td>364 ± 9</td>
<td>100</td>
</tr>
</tbody>
</table>

Mean ± SE or individual values are given. Percentages are based on the initial values at 0.5 hr. Rela-
tionships between PE-32P and $^{14}$C for times between 0.5 and 72 hr are shown in Figs. 1–3.
Fig. 1. Changes in the levels of radioactivity in membrane PE-^{32}P and total RBC-^{14}C after transfusion of doubly-labeled reticulocytes into normal recipient rats. Percentages are based on the initial values at 0.5 hr, as shown in Table 1. Means ±SE are given for four experiments in each of the three experimental groups. Stress reticulocytes were from rats with brisk reticulocytosis induced by hemorrhage. "Iron-deficient reticulocytes" were from rats with severe iron-deficiency anemia associated with ineffective erythropoiesis. PE-^{32}P was measured in red cell ghosts. Total RBC-^{14}C reflects cytoplasmic hemoglobin-^{14}C.
increased to a slightly smaller extent than in the corresponding experiments with intact recipients (Table 1, Fig. 2). With transfusion of stress reticulocytes into splenectomized recipients, there was still disproportionate loss of the membrane label, but the ratio of change was considerably smaller than that observed with normal recipients.

**Experimental Findings for Iron-deficient Reticulocytes**

In agreement with earlier observations, the fall in 14C labeling was much greater with iron deficient reticulocytes than with the other cell types studied (Table 1, Fig. 1). 14C activity fell 23% during the first 4 hr and decreased an additional 28% over the next 3 days. Disproportionate loss of the 32P membrane label was again observed, but the ratio of PE-32P to 14C fell to a much smaller extent and more irregularly than in the corresponding experiments with stress reticulocytes (Fig. 3).

**Validation of Experimental Design**

**Stability of PE-32P as a membrane label.** In vitro experiments confirmed earlier observations that PE is a relatively fixed, nonexchangeable component of the red cell membrane (Table 2). When blood containing 32P-labeled stress reticulocytes was incubated with normal serum for 32 hr, a maximum of 7% of the radioactivity originally present in membrane PE was recovered as...
Table 2. Per Cent Turnover of Membrane Phosphatidylethanolamine (PE)-32P in Stress Reticulocytes During Incubation in Rat Serum

<table>
<thead>
<tr>
<th>Hours of Incubation</th>
<th>Labeled Cells</th>
<th>Unlabeled Serum*</th>
<th>Unlabeled Cells</th>
<th>Labeled Serum†</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>0</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>2</td>
<td>2.0</td>
<td>0.4</td>
<td>0.4</td>
<td>0.4</td>
</tr>
<tr>
<td>4</td>
<td>0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>8</td>
<td>2.0</td>
<td>1.6</td>
<td>1.6</td>
<td>1.6</td>
</tr>
<tr>
<td>16</td>
<td>7.0</td>
<td>2.2</td>
<td>2.2</td>
<td>2.2</td>
</tr>
<tr>
<td>32</td>
<td>5.0</td>
<td>2.2</td>
<td>2.2</td>
<td>2.2</td>
</tr>
</tbody>
</table>

Values are averages of duplicate determinations.

*Per cent turnover of PE calculated as PE-32P radioactivity found in serum divided by radioactivity originally present in membrane PE.
†Per cent turnover of PE calculated from radioactivity found in membrane PE divided by specific activity of serum 32P,.

PE-32P in the serum. Correspondingly, when unlabeled stress reticulocytes were incubated with serum containing a tracer quantity of 32P, the PE-32P isolated from ghost preparations represented a maximum turnover of 2.2% of membrane PE.

Morphology of cell membrane preparations. Electron micrographs of blood from bled donors showed cells with polyribosomes and mitochondria, as would be anticipated with the presence of large numbers of young reticulocytes. Examination of ghosts prepared by hemolysis of the same blood at room temperature failed to reveal these organelles (Fig. 4A). Hemolysis at 4°C produced ghosts less completely free of organelles (Fig. 4B). Correspondingly, virtually all of the red cell lipid present in whole stress reticulocytes was recovered in ghosts prepared at 4°C, whereas 22%–26% was lost when ghosts were made at room temperature.

Light microscopic examination of stress reticulocyte ghosts prepared at room temperature and stained with phosphotungstic acid hematoxylin, Janus green or methyl green pyronin failed to show positive staining. These histochemical findings further confirmed the lack of major contamination of the ghosts with mitochondria or nucleic acid-containing material.

Validity of total 14C counts as a reflection of hemoglobin label. The rate of fall of total red cell 14C was proportionate to that of hemoglobin heme-14C after transfusion of labeled stress reticulocytes (Table 3); although of a different order of magnitude, total red cell 14C is therefore an accurate reflection of hemoglobin heme-14C activity. This is also true for the experiments with iron deficient cells since the observed changes in total 14C activity corresponded very closely to earlier findings10 for 59Fe-labeled cells and for hemoglobin heme-14C conversion to bilirubin-14C. Therefore, since the changes in relationship between cytoplasmic hemoglobin-14C and membrane PE-32P were of importance in these experiments, the simpler determinations of total red cell 14C were employed routinely.

Effects of osmotic hemolysis of whole cells. The findings in the two rats given infusions of distilled water after transfusions of doubly labeled reticulo-
Fig. 4. (A) Electron micrograph of RBC ghosts prepared at room temperature. Note that the layers of membranes (Me) are relatively devoid of content and organelles except for some fibrillar material (f) along the inner surfaces of the ghosts as previously observed by Marchesi and Palade. × 36,000. (B) Electron micrograph of RBC ghosts prepared at 4°C. Note that a number of ghosts contain filamentous material (f), membranous organelles, and occasional free ribosomes (r). Some of the organelles can be identified as distorted mitochondria (arrow) because of their typical double membranes. × 36,000 (courtesy of Dorothy F. Bainton).
Table 3. Comparison of Total RBC-14C and RBC heme-14C in Three Rats Transfused With 14C-labeled Stress Reticulocytes

<table>
<thead>
<tr>
<th>Time after Transfusion (hr)</th>
<th>Total RBC-14C (dpm/10^8 RBC)</th>
<th>RBC heme-14C (dpm/10^8 RBC)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>1524.2 (100%)</td>
<td>385.5 (100%)</td>
</tr>
<tr>
<td>±14.1</td>
<td>±3.0</td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>1464.2 (96.1%)</td>
<td>366.0 (94.9%)</td>
</tr>
<tr>
<td>±299.6</td>
<td>±2.0</td>
<td></td>
</tr>
<tr>
<td>48</td>
<td>1347.2 (88.4%)</td>
<td>347.9 (90.2%)</td>
</tr>
<tr>
<td>±51.0</td>
<td>±3.7</td>
<td></td>
</tr>
<tr>
<td>72</td>
<td>1276.4 (83.7%)</td>
<td>329.4 (85.5%)</td>
</tr>
<tr>
<td>±88.3</td>
<td>±3.6</td>
<td></td>
</tr>
</tbody>
</table>

Means ±SE are shown. Values in parentheses are per cents of initial figures at 0.5 hr.

cytes (Table 1, Fig. 3) confirmed the supposition that hemolysis of whole cells would be associated with proportionate loss of both the membrane and the cytoplasmic labels. The ratio of PE-32P to 14C remained constant during the water infusion from 4-48 hr and thereafter to 72 hr. Hematocrits fell from 45.5 to 27.5 and from 45 to 25.5 in these two animals during the infusion and remained stable for an additional 48 hr, confirming that the anemia was not dilutional in origin. In contrast to the hematocrits, PE-32P and 14C per 10^8 red cells fell only slightly, indicating that the severe osmotic lysis affected labeled and unlabeled cells indiscriminately.

DISCUSSION

Loss of membrane PE-32P exceeded that of red cell 14C in the experiments with normal, stress and iron deficient reticulocytes (Figs. 1–3), although this finding was by far most striking with stress reticulocytes. Disproportionate loss of the membrane as compared to the cytoplasmic label could be explained by three different mechanisms: surface remodeling, selective destruction of a small reticulocyte population which has a marked excess of cell membrane, or loss from intact cells of mitochondria or other ultrastructural components that are rich in membrane lipids. All three mechanisms may be operative to some extent, but the present evidence indicates that the excessive loss of membrane lipid is due primarily to surface remodeling of stress reticulocytes during their maturation in the peripheral blood.

It is possible that some stress cells are destroyed in their entirety. However, quantitative considerations make it improbable that the observed findings were due to the selective loss of young reticulocytes with a particularly high ratio of PE-32P/14C. Such a subpopulation would have to be quite small since hemolysis of a substantial fraction of the transfused cells would have led to a symmetrical loss of the two labels, as when intravascular hemolysis was produced by infusion of water (Fig. 3). Moreover, these few cells would have to contain an enormous excess of membrane lipid if their destruction alone were to account for the dramatic fall in the PE-32P/14C ratio (Fig. 2). These considerations make it much more probable that the loss of membrane lipid emanated from most or all of the stress reticulocytes transfused.

The manner in which PE-32P was assayed in red cell ghosts obviated the
possibility that the observed changes were related to any large extent to the loss of intracellular lipid-containing constituents such as mitochondria or ribosomes. Endoplasmic reticulum can be excluded from consideration because of the paucity of this material in even the most immature erythroid cells. Shattil and Cooper recently described substantial losses of unlabeled membrane lipids, including PE, from whole stress reticulocytes as they matured in vivo. Their evidence suggested that, although part was related to the loss of plasma membrane, part was due to the disappearance of mitochondria from these maturing cells. In the present experiments, however, mitochondria were not found by either histochemistry or electron microscopy in the ghost preparations in which PE-32P was measured. Although we are not aware of such studies for red cells, electron microscopy has proved highly sensitive in detecting mitochondria in other blood elements; in a study of leukocyte fractions, examination of fine structure revealed occasional contaminating mitochondria even though biochemical determination of marker enzymes was negative. Moreover, in the studies of Shattil and Cooper the entire phospholipid content of the whole cells was recovered in the ghost fraction, whereas, in the present experiments, 23% of the original phospholipid was lost during preparation of the ghosts. As shown in Figs. 4A and 4B, the elimination of mitochondria was due to the preparation of cell ghosts at room temperature rather than at 4°C. Bessis (personal communication) has observed that mitochondria escape more readily from reticulocytes subjected to sudden, rather than gradual hemolysis; in the present study, preparation of ghosts at 25°C rather than 4°C may have led to more rapid cell rupture because of increased fluidity of the membrane and contents of the cells.

The conclusion that stress reticulocytes lose excess cell membrane by a process of surface remodeling is in agreement with the findings of Ganzoni et al. The loss of red cell label observed in earlier studies is presumably explained by degradation of fragments of red cell cytoplasm associated with relatively large amounts of plasma membrane rather than by premature destruction of whole reticulocytes. Indeed, just such a budding phenomenon has recently been observed microscopically in reticulocytes embedded in a loose mesh of agar (Gasko and Danon, personal communication). It is possible that by this mechanism macroreticulocytes may achieve normal size and normal life span; however, we did not examine these questions specifically in this study. Moreover, it should be noted that the studies suggesting that stress reticulocytes are short-lived were carried out over several days, whereas the present experiments were confined to the first 72 hr when much of the remodeling is accomplished (Figs. 1 and 2).

The findings in splenectomized recipients (Fig. 2) indicate that the spleen accounts for some, but not all, of the loss of membrane material from stress reticulocytes. Shattil and Cooper also found that splenectomy abolished some of the membrane lipid loss from such cells, although to a smaller extent than in the present experiments. Similarly, with normal reticulocytes, both the magnitude of the changes in membrane PE-32P and red cell 14C and the ratio of these changes fell less in each of the two splenectomized recipients than in any of the four corresponding experiments with intact recipients (Table 1, Fig. 2).
Moreover, in these latter experiments loss of PE-\(^{32}\)P exceeded that of \(^{14}\)C to a small but significant extent (Fig. 1). It is therefore probable that normal reticulocytes also undergo a minor degree of surface remodeling which also is partly mediated by the spleen. Surface remodeling may be an aspect of normal erythroid cell development. This may be largely confined to the bone marrow under physiologic conditions, but, with erythroid stimulation, early macrocytic cells may leave the marrow before the remodeling process has been completed.

In contrast to the role of the spleen in surface remodeling, earlier experiments failed to demonstrate any effect of splenectomy on the rate of disappearance of \(^{59}\)Fe from \(^{59}\)Fe-labeled stress reticulocytes or on the conversion of hemoglobin-\(^{14}\)C in these cells to bilirubin-\(^{14}\)C.\(^{11}\) Similarly, other investigators have reported that splenectomy fails to alter the survival of stress reticulocytes.\(^{36,37}\) Hence, it appears that the spleen may be responsible for the initial phases of the remodeling process in which membrane but not cytoplasm is removed, whereas later events during which some hemoglobin is also lost take place elsewhere, perhaps in the free circulation.\(^{12}\) Another possible explanation is that stress reticulocytes undergo two processes: loss of surface membrane, occurring partly in the spleen, and a comparatively minor degree of true hemolysis and hemoglobin loss, taking place in other sites. Landaw et al. have reported somewhat different results for mice with congenital microcytosis in which reticulocytes do in fact appear to be destroyed in the spleen.\(^{38}\)

Reticulocytes from iron-deficient rats were studied in order to evaluate the fate of immature red cells in a condition associated with pathologic ineffective erythropoiesis.\(^{15}\) It should be noted that iron-deficiency anemia in rats differs in certain respects from this condition in humans and is characterized by reticulocytosis and by a more marked degree of hemolysis of both red cell precursors and circulating erythrocytes.\(^{15,16,39-42}\) In both iron-deficient rats and rats with erythroid hyperplasia stimulated by hemorrhage, there is an increase in the erythropoietic component of early-labeled bilirubin.\(^{11,15,42}\) However, the net production of red cell hemoglobin heme is decreased in iron-deficient rats, whereas it is increased in bled animals;\(^{15,43}\) i.e., there is disproportionate loss of heme pigment per unit of effective hemoglobin synthesis in the former but not in the latter condition. Correspondingly, the rate of hemoglobin degradation is much greater with labeled reticulocytes from iron-deficient donor rats compared to bled donor rats;\(^{11,16}\) indeed, about 50\% of the hemoglobin heme in iron-deficient reticulocytes is lost within 3 days\(^{16}\) (Fig. 1). It was therefore not surprising that the present findings with iron-deficient reticulocytes reflected in large part hemolysis of whole cells; i.e., there was a more or less symmetrical decrease in both the membrane and cytoplasmic labels (Table 1, Figs. 1 and 3). The rapid initial loss of both PE-\(^{32}\)P and \(^{14}\)C that was observed uniquely with iron-deficient reticulocytes is perhaps explained by sequestration and/or destruction of defective cells which may have been rendered more fragile during the transfusion procedure. These observations therefore directly confirm the concept that "ineffective erythropoiesis" in certain pathologic conditions, including iron deficiency anemia,\(^{15,16,39-42}\) is indeed associated with the premature destruction of young erythroid cells.\(^{34,45}\)

Some excess membrane loss was observed in the experiments with iron de-
icient reticulocytes (Fig. 3), suggesting that there had been some surface fragmentation of cells that had escaped hemolysis. There is also evidence from radioautographic studies that some stress reticulocytes are hemolyzed entirely.10,12 Perhaps, then, hemolysis and surface remodeling are parts of a spectrum and remodeling leads to hemolysis when a critical surface area to volume relationship is attained.46,47

The magnitude of the observed decreases in specific activity probably overestimates the actual losses of cellular material. The recipient rats were not in a steady-state; up to 8 ml of blood were removed over a period of 72 hr and the production of new, unlabeled cells may have diluted the labeled cells as the experiments progressed. However, the ratios between PE-32P and 14C should have remained undisturbed. Similar considerations may explain the lack of difference in the decrease of 14C labeling in recipients of stress as compared to normal reticulocytes (Table 1, Fig. 1). In earlier experiments determination of 14C labeling in circulating cells was an insensitive method of detecting small changes, whereas simultaneous measurements of bilirubin-14C excretion demonstrated a significant small increase in hemoglobin-14C loss from stress reticulocytes. Nevertheless, the measurements of red cell 14C in the present experiments were sufficient to detect the relatively large difference between changes in PE-32P and 14C labeling.

These experiments draw attention to the variety of “stress reticulocytes” and implicate two mechanisms for the loss of cellular material from maturing erythroid cells: surface remodeling, as with reticulocytes produced during the normal marrow response to anemia; and hemolysis of whole cells, as with reticulocytes produced by rats with ineffective erythropoiesis related to iron-deficiency anemia. The role of surface remodeling in normal red cell development and the relationship of remodeling to hemolysis of whole reticulocytes in pathologic conditions remain subjects for future investigation.

ACKNOWLEDGMENT

The authors are grateful to Dorothy F. Bainton, M.D., of the Department of Pathology, University of California, San Francisco for the electron micrographs.

REFERENCES

10. Stryckmans PA, Cronkite EP, Biocomelli
SURFACE REMODELING VS. WHOLE-CELL HEMOLYSIS


18. Tarlov AR, Gibson MS: Erythrocyte phospholipids. In vivo turnover in the rat using choline-methyl-3H, choline-methyl-14C and 32P as label. Submitted for publication


47. Weed, RI, Reed, CF: Membrane alterations leading to red cell destruction. Am J Med 41:681, 1966

Surface Remodeling vs. Whole-Cell Hemolysis of Reticulocytes Produced With Erythroid Stimulation or Iron Deficiency Anemia

Steven E. Come, Stephen B. Shohet and Stephen H. Robinson