Independence of Mechanical Fragility and Red Blood Cell Age in the Rat* (34071)

S. I. Shapiro, S. A. Landaw, H. S. Winchell, and M. C. Williams

Department of Chemical Engineering and the Donner Laboratory, University of California, Berkeley, California 94720

The mechanism by which red blood cells (RBC) are eliminated from the circulation in both the normal and diseased states is uncertain. According to Jandl (1), hemolytic processes seem to be the result of metabolic or structural injury, erythrophagocytosis being a secondary rather than a primary event. The possibility that increasing susceptibility to mechanical trauma may be the primary factor limiting the lifespan of RBC prompted this study of the "mechanical fragility" of rat erythrocytes as a function of cell age. In this paper, "mechanical fragility" is defined as the susceptibility of RBC to release hemoglobin into plasma as a consequence of the mechanical trauma imposed on the individual cells.

Materials and Methods. Forty-nine male buffalo rats (approximately 300 g each, Simonsen Laboratory, Gilroy, California) were injected intravenously via a lateral tail vein with 9.5 μCi of ^{59}Fe (ferrous citrate, specific activity 11 mCi/m*M*, Abbott Laboratories, North Chicago, Illinois) under light ether anesthesia. The iron was incubated with sufficient rat plasma prior to injection to assure complete binding of ^{59}Fe to transferrin. After injection of radioiron, approximately 18 mg of elemental iron were injected subcutaneously every 2–3 days (Imferon, Lakeside Laboratory, Milwaukee, Wisconsin; 50 mg of elemental iron per cc) to minimize reutilization of ^{59}Fe.

On a given day after the ^{59}Fe injection, two of the rats were anesthetized with ether and exsanguinated by aortic puncture into a heparinized syringe. The hematocrit of this blood was determined using a standard micro method, and total blood hemoglobin was determined by the cyanmethemoglobin method. Total blood radioactivity was established by lysing 0.1 ml of whole blood in saponin and counting the resulting solution in an automatic well counter.

The rest of the blood was mildly centrifuged (500*g* for 5 min) permitting withdrawal of plasma to adjust the hematocrit to approximately 0.50, in order to maintain standard conditions for the subsequent shearing experiment. The supernatant plasma removed was used to determine the concentration of ^{59}Fe and hemoglobin in the plasma prior to the application of the shearing force. Plasma hemoglobin was determined by the benzidine method of Crosby and Furth (2).

After a delay of about 45 min from the time of blood removal from the rat, 4.75 ml were pipetted into the gap spaces of a concentric cylinder Couette viscometer (Haake Rotovisco, Brinkman Instruments, New York, New York; See Fig. I). The rotating bob was accelerated in 45 sec to the speed under consideration (1300 rpm), corresponding to shear stresses of approximately 325 dynes/cm^2, imposed uniformly throughout the fluid contained in the gap spaces. This shearing was maintained for 2 min, after which time the rotational speed was gradually reduced to zero. A circulating water bath maintained the temperature at 30.5 $\pm$ 0.5°. After application of the shearing stress, the blood was poured from the assembly into a test tube. The hematocrit was determined, the remaining blood centrifuged (500*g* for 10 min), and the supernatant plasma with-

* This study was supported, in part, by a grant-in-aid from the Bay Area Heart Association, and also by the U. S. Atomic Energy Commission and the National Aeronautics and Space Administration, under Contract W-7405-eng-48.

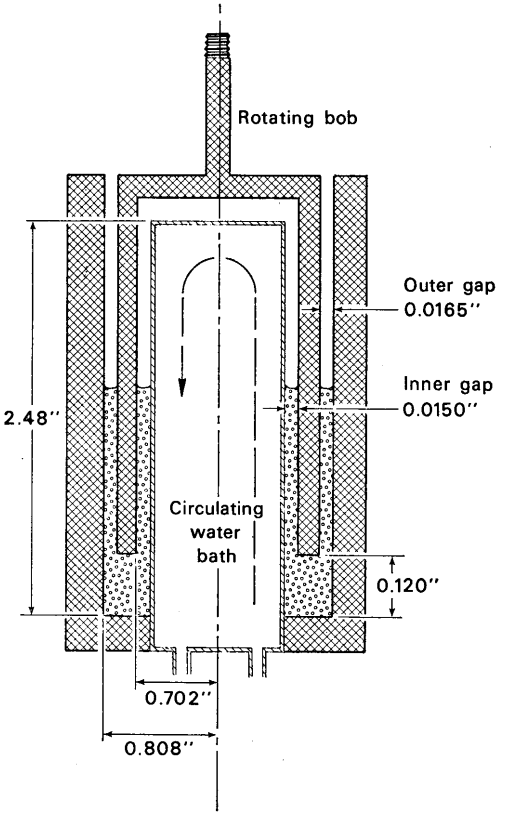

FIG. 1. Double gap concentric Couette viscometer used for the shearing experiments. The standardized shearing conditions were: rpm, 1300; shear rate, 6500 sec^{-1}; shear stress, 325 dynes/cm^2; temperature, 30.5°.

drawn. This solution was analyzed for hemoglobin and ^{59}Fe activity by the same methods previously noted.

Red blood cell ^{59}Fe specific activity was determined by subtracting plasma activity from total blood activity for all rats studied, and the resultant "corrected" radioactivity in the RBC was expressed as DPM per gram of hemoglobin. The data points of RBC hemoglobin specific activity versus time after ^{59}Fe injection were fitted to an equation of appropriate form (3, 4) using a least-squares minimization fitting program and a CDC-6600 digital computer.

The percentage of RBC hemoglobin released into the plasma due to controlled shearing is,

$$\% \text{ Hgb released} = \frac{\text{mg Hgb in final plasma} - \text{mg Hgb in initial plasma}}{\text{total mg Hgb in RBC}} \times 100 \quad (1)$$

The percentage of RBC ^{59}Fe activity released was calculated from the following formula:

$$\%\ ^{59}\text{Fe released} = \frac{\text{DPM in final plasma} - \text{DPM in initial plasma}}{\text{total DPM in RBC}} \times 100 \quad (2)$$

The ratio of percentage of ^{59}Fe released to percentage of hemoglobin released (R), was also calculated for each rat studied:

$$R = \frac{\%\ ^{59}\text{Fe released}}{\%\ \text{Hgb released}} \quad (3)$$

The curve of this ratio R versus time after ^{59}Fe injection was analyzed by standard statistical tests, including a linear regression analysis.

Results. The body weights of the animals remained constant throughout the study. The percentage of hemoglobin released from the RBC by the mechanical shear forces used (Eq. 1) was relatively constant, and generally fell within the range of 0.2–0.7%.

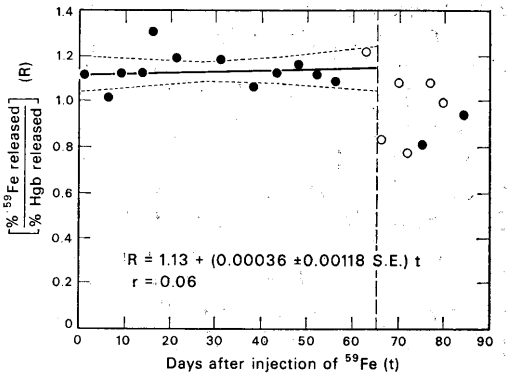

FIG. 2. Curve of the ratio R (% ^{59}Fe released/ % Hgb released) versus time after injection of ^{59}Fe (days). The closed circles represent the average of at least two animals, while the open circles represent results in a single animal. The solid line represents the least squares "best fit" of a linear regression line through the data points taken from 1–65 days after ^{59}Fe injection. The dotted lines above and below this regression line indicate the 95% confidence limits for this line (r = correlation coefficient).

The ratio, R, representing the "mechanical fragility" of the cohort of labeled RBC relative to that of the entire RBC population, is plotted in Fig. 2 versus the time after radioiron injection. Each data point represents the average of the results obtained for the blood of the animals studied that day. The solid line represents the least-squares best fit of the data points to a linear regression line, while the dotted lines above and below the solid line represent the 95% confidence limits of this regression line. The "best fit" value for the intercept of this line was 1.13, with a slope of 0.000360 ± 0.00118 (SE).

Discussion. Red blood cell survival, as determined from RBC ^{59}Fe specific activity (Table I) was similar to that obtained by Belcher and Harriss (4). However, the estimated mean potential lifespan of the cohort of RBC is about 10% less than that measured in rats using the heme-^{14}C (5) or ^{14}CO techniques (6). This latter discrepancy may be related to the chronic inflammation at the multiple sites of iron dextran injection almost invariably seen when using the present technique.

In Fig. 2 the constancy of the ratio of percentage of ^{59}Fe released to percentage of Hgb released after shearing during the period of 1–65 days after cohort labeling is evidence for lack of dependency of shear-induced hemolysis on RBC age. The slope of the curve of the regression line for this ratio, R, with time after ^{59}Fe injection, 0.000360 ± 0.00118 (SE) is not significantly different from zero. The intercept of 1.13 differs by 13% from the theoretical ratio of 1.0 expected if the "mechanical fragility" of the cohort is exactly the same as that of the entire RBC population. This small difference may be due to certain consistent errors involved in the determination of this ratio. Since the cohort of RBC can be considerred to have lasted only about 65 days in these experiments, data points obtained after this time cannot be considered as representing "mechanical fragility" of cells of known age. The wide scatter of the points obtained after 65 days may represent abnormalities in RBC properties due to the chronic inflammation seen in the animals at these later times.

TABLE I. Least Squares "Best Fit" Results for the Parameters of RBC Survival Derived from Red Blood Cell ^{59}Fe Specific Activity.

Parameter	Value
Rate of random hemolysis	0.0097/day
Mean potential lifespan	59.6 days
Coefficient of uniformity of lifespan	0.194 days
Maximum Hgb specific activity	4.71×10^6 DPM/g
Fractional ^{59}Fe reincorporation	16.6%

The mechanism(s) by which the hemoglobin was released from the RBC during the shearing is uncertain. There is evidence, however, that hemolysis involved interaction between the cell and the viscometer surfaces (7). On examination of stained films of the sheared blood, no cell fragmentation could be observed, and no cell ghosts were seen using phase microscopy.

A previous investigation of "mechanical fragility" as a function of cell age was conducted by Stewart *et al.* (8) using dog blood and the rolling glass beads technique of Shen, Castle, and Fleming (9). In their first two experiments, they neglected to subtract initial plasma ^{59}Fe activity and there was a tenfold variation in the values of percentage of RBC hemoglobin released. In their third experiment, the values of percentage of RBC hemoglobin released were constant, and by washing the RBC prior to imposing the trauma, the correction for initial plasma ^{59}Fe activity was not necessary. The results of the third experiment agreed well with those of the present investigation—namely that "mechanical fragility" is relatively independent of RBC age.

If metabolic or structural injury to RBC precedes and induces erythrophagocytosis, and if such injury were simulated by the imposition of mechanical "hemolytic forces," one would anticipate that the measurement of "mechanical fragility" in the present study would have increased with cell age. That such was not the case indicates that the ability to resist the type of mechanical trauma used in the present experiment is unrelated to cell age and the associated phenomenon of senescent destruction.

Summary. Whole blood of rats was sub-

jected to a standardized amount of mechanical trauma (325 dynes/cm^2 for 2 min) after the production of cohorts of ^{59}Fe-labeled red blood cells, and the relative release of hemoglobin and ^{59}Fe from the red blood cells was determined. The results indicate that rat red blood cell susceptibility to mechanical hemolysis is independent of cell age.

The authors are indebted to Barbara Shipley, Kay Stoddard, and Rosanne Raley for their invaluable technical assistance.

1. Jandl, J. H., Am. J. Med. **41**, 657 (1966).

2. Crosby, W. H. and Furth, F. W., Blood **11**, 380 (1956).

3. Eadie, G. S. and Brown, I. W., Blood **8**, 1110 (1953).

4. Belcher, E. H. and Harriss, E. B, J. Physiol. **146**, 217 (1959).

5. Berlin, N. I., Meyer, L. M., and Lazarus, M., Am. J. Physiol. **165**, 565 (1951).

6. Landaw, S. A., Donner Laboratory Semiannual Report, UCRL-17481 (1967).

7. Shapiro, S. I. and Williams, M. C., Am. Inst. Chem. Eng. J. (in press).

8. Stewart, W. B., Stewart, J. M., Izzo, M. J., and Young, L. E., J. Exptl. Med. **91**, 147 (1950).

9. Shen, S. C., Castle, W. B., and Fleming, E. M., Science **100**, 387 (1944).

Received Jan. 21, 1969. P.S.E.B.M., 1969, Vol. 131.