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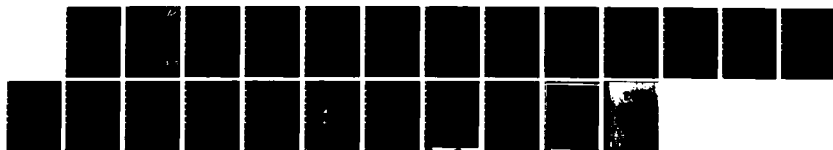
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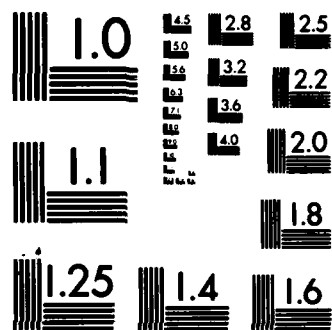
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THE RELATIONSHIP BETWEEN ERYTHROCYTE VOLUME AND CELL AGE
IN HUMANS AND BABOONS

by

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To verify the biochemical evidence that erythrocytes decrease in volume with aging, in vivo cohort labeling of red blood cells with ^{59}Fe was performed in baboons. A similar relationship of EGOT to Hb was observed to that in the human subpopulations. The peak activity of $^{59}\text{Fe}/\text{RBC}$ appeared initially in the red blood cells with the highest MCV and progressed from the erythrocytes with the largest MCV to the erythrocytes with the smallest MCV over the next 10-12 weeks. While a certain amount of erythrocyte volume heterogeneity seems to be present as a result of erythropoiesis, our data support the hypothesis that red blood cells decrease in volume as they age.

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ABSTRACT

The relationship of red blood cell size to age during steady-state hematopoiesis has been studied using erythrocytes separated on the basis of size using counterflow centrifugation. Human red blood cells with an original mean corpuscular volume (MCV) of $89.2 \pm 4.1 \mu^3$ were isolated, free of plasma protein and other cell contaminants, into 7 fractions ranging in size from $77.0 \pm 2.7 \mu^3$ to $98.5 \pm 4.8 \mu^3$. The ratio of the age-related enzyme, erythrocyte glutamic oxaloacetic transferase (EGOT), to hemoglobin (Hb) increased progressively through the fractions, suggesting a correlation between erythrocyte volume and age. Reticulocytes, while present in all fractions, were selectively enriched in the larger subpopulations.

To verify the biochemical evidence that erythrocytes decrease in volume with aging, in vivo cohort labeling of red blood cells with ^{59}Fe was performed in baboons. A similar relationship of EGOT to Hb was observed to that in the human subpopulations. The peak activity of $^{59}\text{Fe}/\text{RBC}$ appeared initially in the red blood cells with the highest MCV and progressed from the erythrocytes with the largest MCV to the erythrocytes with the smallest MCV over the next 10-12 weeks. While a certain amount of erythrocyte volume heterogeneity seems to be present as a result of erythropoiesis, our data support the hypothesis that red blood cells decrease in volume as they age.

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INTRODUCTION

While red blood cells are known to be heterogenous in size, the etiology of the erythrocyte volume distribution remains controversial.¹⁻³ The size of reticulocytes produced by the bone marrow is usually proportional to the degree of erythropoietic stimulation.^{4,5} Although reticulocytes have been shown to undergo fairly rapid size reduction⁶⁻⁸ associated with organelle loss and membrane processing, the magnitude and duration of continued size reduction of erythrocytes has not been fully evaluated. Recent evidence by Weiser and Kociba³ suggests that the initial size of a reticulocyte is a determinant of the mature erythrocyte size. These authors have suggested that the production of reticulocytes under different degrees of erythropoietic stimulation may determine the heterogeneity of the erythrocyte volume distribution.

In contrast, density-defined subpopulations have demonstrated correlations between erythrocyte density and age,⁹⁻¹² and between erythrocyte density and size.^{13,14} Based on these data, an indirect correlation between erythrocyte size and age has been drawn. To date, no direct study of the relationship between erythrocyte size and age has been performed.

To examine the relationship between erythrocyte volume and age, counterflow centrifugation was used to fractionate red blood cells into subpopulations on the basis of size. We have examined the relationship of erythrocyte size to an age-related enzymatic activity (EGOT), reticulocyte distribution in humans, and ⁵⁹Fe kinetics in baboons.

MATERIAL AND METHODS

Erythrocyte Subpopulations

The basic methodology for isolation of size-dependent cell subpopulations by counterflow centrifugation has been described previously.¹⁵ In brief, counterflow centrifugation opposes an outwardly directed centrifugal force with an inwardly directed force generated by the flow of fluid through the centrifuge separation chamber. Cells of different size equilibrate at different positions in the chamber and can be removed from the centrifuge sequentially by increasing the rate of flow through the chamber. Separation is achieved on the basis of cell size and the effect of differing cell density is minimal.¹⁵

The red blood cell donors were healthy laboratory personnel, 11 males and 4 females, between the ages of 19 and 49. Ten ml of blood were collected and anticoagulated with sodium heparin. Eight hundred μ l of the blood were diluted into 10 ml of elutriation buffer (125 mM NaCl, 2.6 mM KCl, 8.1 mM Na_2HPO_4 , 1.4 mM KH_2PO_4 , 50 mM d-Glucose, and 0.5% bovine serum albumin adjusted to $\text{pH}=7.40\pm0.05$, osmolarity 305 ± 5 m Osm). The red blood cells were separated into 7 subpopulations using a Beckman Centrifuge No. J21B with an elutriation rotor (JE6) and a Sanderson cell separation chamber (No. 335206). All separations were performed in elutriation buffer at 20° C and 2000 rpm. The subpopulations were separated as follows: 2.5 ml of the diluted cell suspension were loaded into the chamber and the stopcock flushed with 2 ml of buffer. The blood

sample was allowed to flow into the chamber at a rate of 4 ml/min. Once in the chamber, the cells were allowed to equilibrate for 10 minutes to allow complete elution of plasma proteins and platelets. Seven sequential fractions were then collected at flow rates of 7, 8, 9, 10, 11, 12, and 13 ml/min for 5 minutes each.

Cell Counting and Sizing

The red blood cells in each fraction and the original diluted sample were counted electronically in triplicate on a Coulter ZBI counter (Coulter Electronics, Hialeah, Fla.). The RBC's were sized on a linear scale using a Coulter ZB counter with an H4 channelyzer attachment and a 50 μ cylindrical aperture. Cells were sized in isotonic PBS and only particles whose size range at this setting was 30-120 μ^3 were used in the sizing data.

Reticulocytes

Cells from each fraction were pelleted by centrifugation and resuspended in 1 ml of autologous serum. The cells were stained with new methylene blue and smears were prepared. The number of cells containing stained granules or reticulum in a sample of 1,000 cells was determined and expressed as a percentage.

Erythrocyte Hb Levels and EGOT Activity

A known volume of each fraction and the unfractionated dilute whole blood were centrifuged at 1500 g x 15 min and the supernatant removed. The cells were resuspended in a volume of triple distilled water

calculated to give a final concentration of 100,000 cell equivalents/ μ l. The fractions were then vortexed and the lysate measured for hemoglobin and EGOT levels. Hemoglobin was measured using the cyanmethemoglobin technique as previously described.¹⁶ EGOT activity was assayed by measuring the conversion of NADH to NAD at 340 nm in the presence of oxaloacetic acid. All measurements were performed in duplicate and the average used in data analysis.

Erythrocyte Density Studies

The mean densities of small erythrocytes (Fractions 1-3), intermediate erythrocytes (Fractions 4 and 5), large erythrocytes (Fractions 6 and 7), and unfractionated erythrocytes were measured by the method of Danon and Marikovsky.¹⁷

⁵⁹Fe Kinetics

Three adult baboons that had not been bled for at least 6 weeks prior to study were used. Animals were housed in conformance with NIH guidelines and fed a routine diet. Animals were anesthetized with ketamine hydrochloride during blood drawing. On the first day of study, a baboon was injected with 50 μ Ci of Iron-59 Citrate IV (New England Nuclear, Boston, Mass.). Seven days after injection, and then at 1-2 week intervals, 10 ml blood samples were drawn for elutriation by direct puncture of femoral vein and anticoagulated with sodium heparin. Baboons were given 1 ml of Imferon (Merrell-National Laboratory, Cincinnati, Ohio) IM weekly to prevent reutilization of ⁵⁹Fe. Red cell

fractionation into size-dependent subpopulations was performed as above. Each subpopulation and the original unfractionated erythrocyte suspension were counted for gamma radioactivity and RBC count and the data expressed as counts per minute/RBC. Erythrocyte EGOT and hemoglobin levels were performed as above.

RESULTS

Using the counterflow centrifuge, reproducible size separation of red blood cells could be achieved (Table 1). The MCV of the fractions ranged from 77.0-98.5 μ^3 . The distribution of red cells in the different subpopulations approximated the frequencies of red blood cells of that size in the unfractionated population. Of the erythrocytes loaded into the separation chamber, 89.7 $\pm$ 7.3% were recovered in the seven subpopulations. If flow rates up to 15 ml/min were used to collect even larger cells, an additional 2.9 $\pm$ 2.8% (n=9, mean $\pm$ S.D.) of the loaded erythrocytes could be recovered. While these cells were enriched for reticulocytes, they also had a significant contamination with lymphocytes. Because of this contamination, these cells were not routinely studied.

Analysis of the age-related enzyme, EGOT, revealed a progressive increase in the ratio of EGOT to hemoglobin through the fractions (Table 1). No statistically significant differences in the mean corpuscular hemoglobin (MCH) were seen between the fractions. The percentage of reticulocytes increased through the fractions in close correlation with

their size. However, none of the subpopulations was significantly enriched for reticulocytes compared to the unfractionated population, confirming as mentioned above that many reticulocytes were excluded from our subpopulations along with leukocytes. Mean densities between the fractions differed by less than 1%.

To confirm the significance of the relationship of age to erythrocyte volume, as suggested by the EGOT data, the distribution through the fractions of the cohort label ^{59}Fe was studied over time in baboons. Although a baboon's erythrocytes are slightly smaller than a human's, they could be easily separated into seven size-dependent subpopulations (Table 2). The distribution of red cells in the different subpopulations was similar to that of human erythrocytes. EGOT activity increased through the fractions by greater than twofold. One week after baboons were injected with ^{59}Fe , activity appeared predominantly in the larger subpopulations (Figures 1, 2 and 3). Over the next 10 weeks of study, the peak activity moved progressively through the smaller subpopulations and by week 11 the peak activity was in Fraction 1. Whole blood ^{59}Fe activity remained relatively constant between weeks 2 and 10, after which it fell off. By week 16 no differences in the activities of any of the fractions were noted. Red cell count and hemoglobin levels remained constant over the course of study.

DISCUSSION

The relationship of mature erythrocyte size and age has been examined during steady-state erythropoiesis. In both humans and

baboons a close correlation between EGOT activity and MCV has been observed. Since Bartos and Desforges¹⁸ have shown a close correlation between EGOT activity and the age of an erythrocyte, these data suggest that the MCV of a mature erythrocyte correlates with its age. As the range of the MCV's of our size-dependent subpopulation encompasses greater than 90% of the cell volumes of mature erythrocytes,¹⁹ it is possible that aging may account for the etiology of the entire erythrocyte volume distribution. Data from the ⁵⁹Fe studies in baboons tend to confirm this statement. If one assumes that a cohort of cells has been labeled, which seems reasonable since the survival time of the label in the circulation closely approximates previously reported survival times for baboon red blood cells,²⁰ then the number of labeled cells in Fraction 7 is greatest one week after labeling and decreases progressively over the lifespan of the cohort (Figures 1, 2 and 3). Simultaneously, the number of labeled cells in the smallest sized fraction (Fraction 1) is least after one week and increases progressively over the lifespan of the cohort. Intermediate fractions tend to peak at intermediate time points and then decline. These data are consistent with a labeled cohort of cells that are decreasing in size over time.

It is important to remember that these studies were performed in subjects whose hemoglobin, hematocrit, and reticulocyte counts were within normal limits and are presumed to be in steady-state erythropoiesis. Reticulocyte size heterogeneity undoubtedly occurs as

noted by the reticulocyte distribution in the fractions. Under conditions of enhanced erythropoiesis, production of reticulocytes with an increased MCV will undoubtedly have significant impact on the red cell volume distribution as demonstrated by Weiser and Kociba.³ However, the normal size range of mature erythrocytes seems to be accounted for by continued size reduction of erythrocytes as they age. Our data provide direct evidence that the cell volume of the mature erythrocyte decreases with age as measured by enzyme content and ⁵⁹Fe kinetics. This confirms the indirect correlation of erythrocyte size and age that has been made using density-defined red blood cell subpopulations. The future study of the biophysical properties of erythrocytes separated on the basis of size may provide important insights into the mechanisms of red cell senescence.

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Table 1: Human Erythrocyte Subpopulations

The mean corpuscular volume, percent of the original erythrocyte suspension, EGOT content, and % reticulocyte count of the 7 fractions and the original erythrocyte suspension. Average recovery of the erythrocytes in the fractions was $89.7 \pm 7.3\%$ (Mean $\pm$ SD).

Fraction No.	Mean MCV (μ^3 , n=15)	% of Recovered Cells (n=15)	EGOT Activity (IU/g Hb, n=6)	Reticulo-cytes (% n=6)	MCH (pg/cell, n=5)
1	77.0 ± 2.7	2.7 ± 1.8	1.9 ± 0.8	0.2 ± 0.1	29.6 ± 2.0
2	81.0 ± 3.7	8.0 ± 4.3	2.0 ± 0.7	0.3 ± 0.1	27.9 ± 3.9
3	84.9 ± 3.3	18.3 ± 7.9	2.5 ± 0.7	0.6 ± 0.1	27.9 ± 3.5
4	89.1 ± 4.0	26.2 ± 4.7	3.2 ± 0.7	0.8 ± 0.2	27.1 ± 4.2
5	92.9 ± 3.8	24.8 ± 5.9	3.8 ± 0.6	0.9 ± 0.1	31.4 ± 4.2
6	95.4 ± 4.6	14.5 ± 7.8	4.4 ± 0.5	1.2 ± 0.3	30.9 ± 7.6
<u>7</u>	<u>98.5 ± 4.8</u>	<u>5.2 ± 4.3</u>	<u>4.9 ± 1.5</u>	<u>0.9 ± 0.3</u>	<u>31.2 ± 4.1</u>
unfraction- ated erythrocytes	89.2 ± 4.1	_____	3.9 ± 0.6	1.0 ± 0.2	29.5 ± 3.6

Table 2: Baboon Erythrocyte Subpopulations

The mean corpuscular volume, percent of the original erythrocyte suspension, and EGOT content of the 7 fractions and the original erythrocyte suspension. Average recovery of the erythrocytes in the fraction was $91.8 \pm 8.5\%$ (Mean $\pm$ SD).

<u>Fraction No.</u>	<u>Mean MCV (μ^3, n=32)</u>	<u>% of Recovered Cells (n=32)</u>	<u>EGOT Activity (IU/gHb, n=5)</u>
1	73.3 $\pm$ 4.4	3.55 $\pm$ 2.19	4.3 $\pm$ 0.8
2	78.8 $\pm$ 5.6	11.66 $\pm$ 6.83	4.8 $\pm$ 1.3
3	81.6 $\pm$ 4.4	22.04 $\pm$ 9.98	5.0 $\pm$ 0.8
4	86.0 $\pm$ 5.2	26.23 $\pm$ 6.17	5.8 $\pm$ 0.9
5	89.1 $\pm$ 5.6	20.14 $\pm$ 7.75	6.3 $\pm$ 0.8
6	92.8 $\pm$ 6.7	11.67 $\pm$ 8.40	7.1 $\pm$ 0.3
<u>7</u>	<u>94.8 $\pm$ 7.1</u>	<u>4.71 $\pm$ 4.45</u>	<u>8.8 $\pm$ 1.5</u>
unfractionated erythrocytes	83.3 $\pm$ 3.7	<u> </u>	5.6 $\pm$ 1.1

FIGURE 1

Distribution of ^{59}Fe over time in baboon red blood cells fractionated on the basis of size.

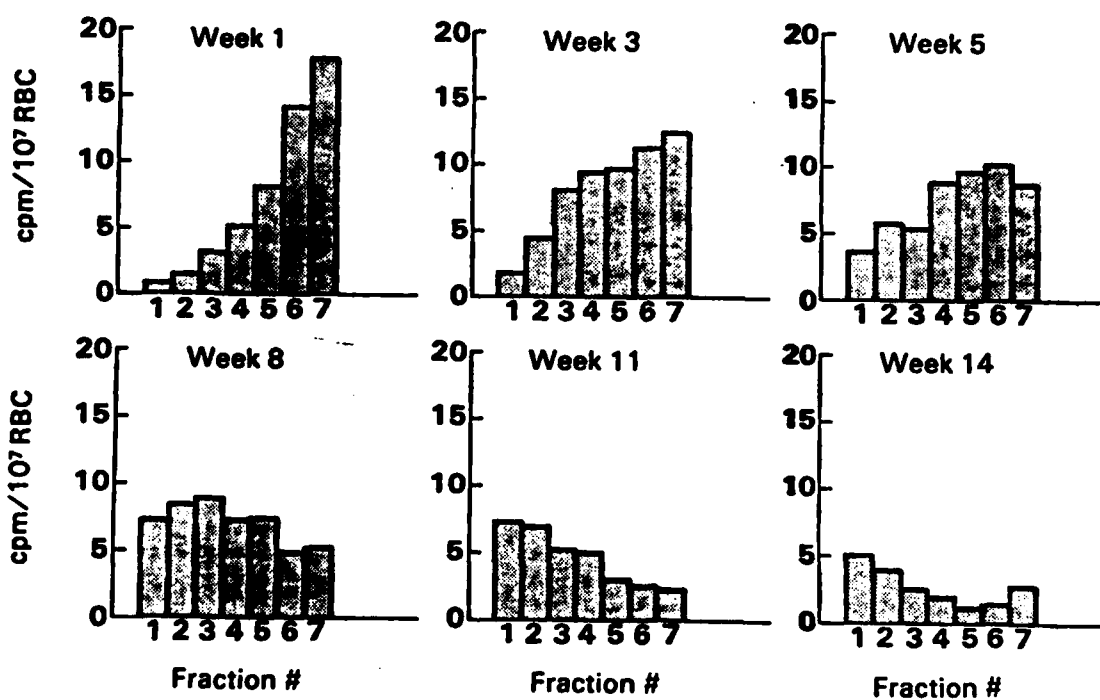


FIGURE 1

FIGURE 2

Distribution of ^{59}Fe over time in baboon red blood cells fractionated on the basis of size.

Baboon 23-81

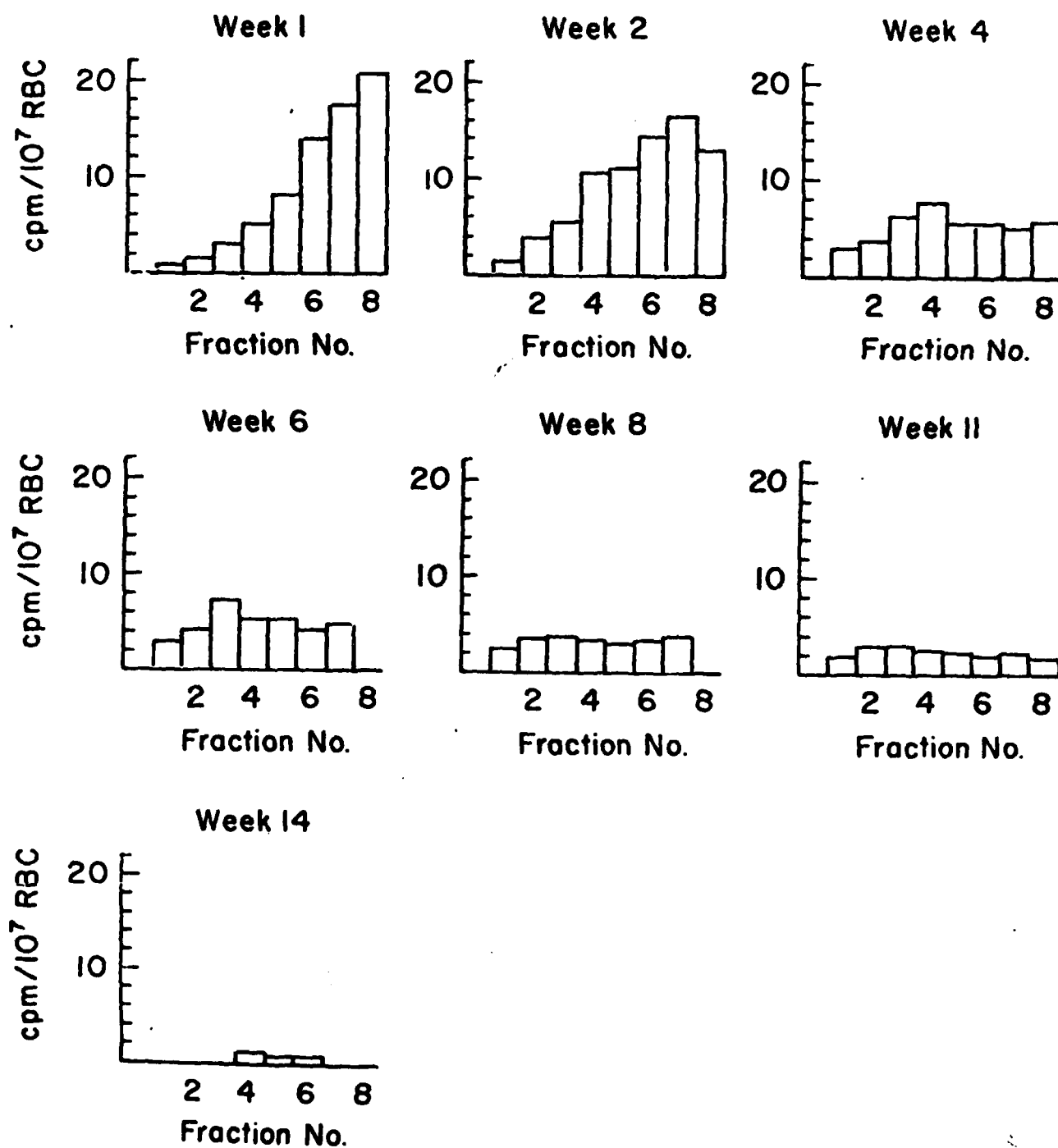


FIGURE 2

FIGURE 3

Distribution of ^{59}Fe over time in baboon red blood cells fractionated on the basis of size.

Baboon 89-78

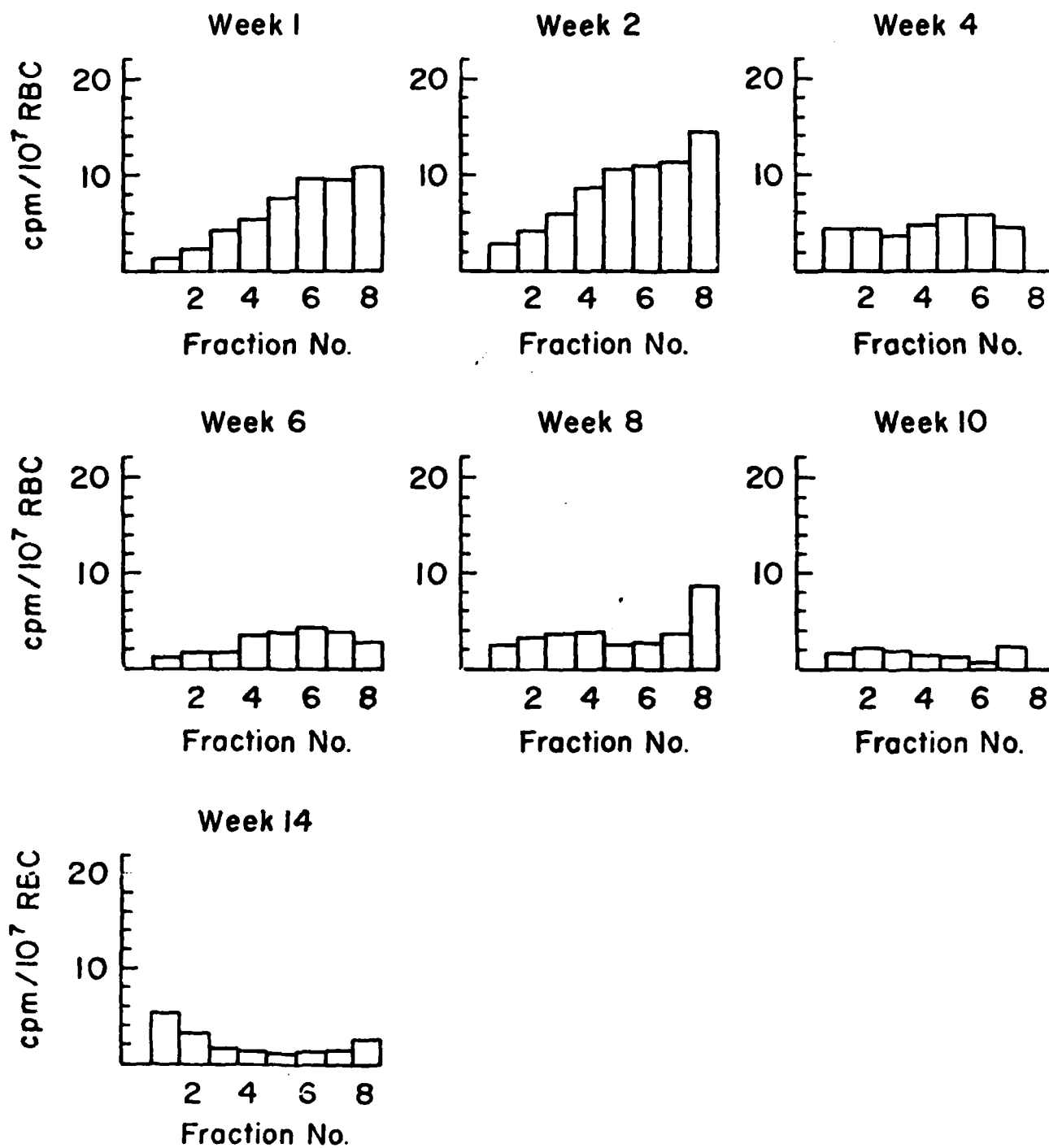


FIGURE 3

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