

Characteristics of Marrow Production and Reticulocyte Maturation in Normal Man in Response to Anemia

ROBERT S. HILLMAN

From the Department of Medicine, University of Washington School of Medicine, Seattle, Washington 98104

ABSTRACT Erythropoiesis in normal man was studied during periods of phlebotomy-induced anemia of varying severity. This study permitted a comparison of marrow production measurements over a wide range of marrow production levels. As long as the serum iron remained above 50 $\mu\text{g}/100\text{ ml}$, measurements of plasma iron turnover provided an excellent index of marrow production at all levels of red cell production. In contrast, the absolute reticulocyte count demonstrated a poor correlation with the other measurements. This was shown to be the result of a prolongation of the time required for circulating reticulocytes to lose their reticulum, which correlated with the severity of the anemia. For the clinical application of the reticulocyte count as a measurement of marrow production, an adjustment must be made for this alteration in the circulating reticulocyte maturation time.

INTRODUCTION

Erythropoiesis in man has been studied by a number of techniques, including: the reticulocyte count, plasma iron turnover, ^{51}Cr or diisopropylfluorophosphate (DF^{32}P) red cell life-span, and stercobilinogen excretion. Except for the reticulocyte count, these measurements are in essential agreement and apparently correlate with the actual level of erythroid marrow production. However, this conclusion is based primarily on measurements in pathological states (1-5). Studies of the normal marrow have been restricted to characterization of basal production and the acute response of marrow to sudden anoxia, phlebotomy, or transfusion polycythemia (1-3, 6-10). As for the reticulocyte count, it has generally been discarded because of a poor correlation with the other production measurements (11, 12).

The present study has examined the response of the

normal human erythroid marrow to graded levels of anemia maintained for 3-5 wk. This examination permitted a comparison of erythropoietic measurements over a wide range of marrow production levels and a definition of the changes in reticulocyte maturation which are important to the interpretation of the reticulocyte count as a production measurement.

METHODS

All studies were performed on the Clinical Research Center of the King County Hospital, Seattle, Washington. Participants included two patients with hemochromatosis, who required prolonged phlebotomy therapy, and six normal volunteers. The subjects were males between the ages of 23 and 48. All studies were carried out according to the guidelines of the Helsinki Declaration for participation of human volunteers. Before study, each individual was carefully evaluated to exclude renal impairment or other significant illnesses which could have interfered with maximum erythropoietin output and marrow response to phlebotomy. During an initial 10 day observation, base line studies of marrow function were obtained, including two measurements of plasma iron turnover, a marrow iron transit time, serum iron, and total iron binding-capacity determinations, a ^{51}Cr -labeled red cell mass, mean cell indices, repeated reticulocyte counts, and a bone marrow examination with Prussian blue stain for iron stores. Throughout the control and subsequent study periods, the subjects were kept on a high protein diet, and folic acid was provided in excess, 5 mg twice a day orally. In order to investigate the role of iron in the marrow production response, iron was provided by varying methods when required (13).

After completion of base line studies, the subjects were phlebotomized, letting 500-1000 ml of whole blood to lower the hematocrit to a level of 32-37%. The total blood volume was kept constant by immediate infusion of an equal quantity of 5% albumin solution. Phlebotomies of more than 500 ml were accomplished in two stages by complete replacement of the plasma volume after removal of each 500 ml. Over the next 3-5 wk, the hematocrit was maintained at this level by graded daily phlebotomy adjusted to remove enough red cells to compensate for increased production. An equal volume of 5% albumin solution was infused with

Received for publication 20 June 1968 and in revised form 4 November 1968.

any phlebotomy of more than 50 ml of whole blood.¹ The erythroid marrow production response was then monitored with twice daily reticulocyte counts, measurements of the plasma iron turnover at 7-day intervals, and from the level of daily phlebotomy required to maintain the hematocrit at this level (see below, phlebotomy production measurement).

With the hematocrit maintained at 32–37%, the erythroid marrow production response was measured in eight individuals, six normals relying on either reticuloendothelial cell iron stores and (or) orally administered iron, and two hemochromatosis patients with excessive parenchymal and reticuloendothelial cell iron stores. Patients on orally administered iron received a 300 mg ferrous gluconate tablet at 2-hr intervals while awake for a total of 8–9 doses/day. Marrow production responses were monitored until production had plateaued for at least 10 days. This plateau was considered to represent a maximum level of marrow production for the selected conditions of anemic stress and iron supply.

Subsequently, six of the normal volunteers and one subject with hemochromatosis were studied at a hematocrit level of 25–30%, maintained over a 3–5 wk period. This level of phlebotomy-induced anemic stress was tolerated by the subjects without difficulty. One individual worked full time cleaning and steaming Alaskan King crab; the others continued their normal daily activities without restriction.

Marrow production studies

Plasma iron turnover measurements. All determinations of the plasma iron turnover were performed at 10:00 a.m. in a fasting state. With patients on orally administered iron the radioiron was injected 1 hour after the last oral dose. 1–10 μ Ci of ferrous citrate-⁵⁹Fe was incubated with 5–10 ml of the patient's plasma for 30 min before injection. The injected dose and standard dilution were determined gravimetrically. Accurately timed venous samples were obtained at frequent intervals over the next 2 hr, immediately chilled

to 0–4°C, centrifuged, and the separated plasma was counted in a gamma-well counter² to 10,000 or more counts above background for $\pm 1\%$ accuracy. The plasma iron turnover was calculated according to Bothwell and Finch (14) with the mean of two serum irons drawn at 0 time and 30 min after radioiron injection and the $t_{1/2}$ clearance. The radioiron administered to any single patient was limited to a maximum of 25 μ Ci for the entire study.

The plasma iron turnover production index was calculated according to Giblett et al. (1) with 0.65 mg/100 ml of whole blood per 24 hr as normal.

$$\text{PIT production index} = \frac{\text{Patient's PIT}}{0.65}$$

In order to study the effect of varying levels of reticulocytosis on the plasma iron turnover measurement, direct reticulocyte uptake of radioiron was measured with each plasma iron turnover by *in vitro* incubation of the patient's blood with ⁵⁹Fe-labeled transferrin. Whole blood was collected in heparin, centrifuged, and the plasma removed. After adjusting the plasma iron to 200–250 μ g/100 ml with ferrous citrate-⁵⁹Fe, sufficient plasma was recombined with the red cell fraction to obtain a hematocrit of 50–60%. Once a zero time sample was obtained the red cell suspension was incubated in a water bath at 37°C. Samples were removed at 60 and 120 min, immediately chilled to 0–4°C, centrifuged, and plasma aliquots obtained for counting. The red cell fraction was washed three times in cold saline, lysed in distilled water, and an aliquot counted for radioactivity. Red cell iron uptake was calculated from the serum iron and the net gain in activity of the red cell fraction over time zero.

Phlebotomy production measurements. Production was also estimated from the level of daily phlebotomy, because if the hematocrit is kept at a constant level, red cell production must equal the amount of packed red cells removed by phlebotomy plus the amount of red cells dying each day.³

¹ 5% albumin solution, Albumisol (Merck, Sharp & Dohme, West Point, Pa.) was administered in equal volumes for any phlebotomy exceeding 50 ml of whole blood. Repeated blood volumes (⁵¹Cr-labeled red cell mass and (or) radioiron-labeled transferrin plasma volume) at weekly intervals confirmed the fact that this technique kept the blood volume constant throughout the periods of prolonged phlebotomy.

Week.....	Hct 32–37%					Hct 25–30%			
	1	2	3	4	5	6	7	8	9
Patient 1	5150	5200	5400	5210	5050	5320	5180	—	5060
2	3780	3800	3655	—	3850	4050	3780	3820	3650
3	4320	4550	4450	4300	4610	4400	—	4510	4480

* Nuclear-Chicago gamma-well counter, Nuclear-Chicago Corporation, Des Plaines, Ill.

³ The amount of red cells lost from senescence each day was obtained by daily calculation of the red cell age distribution as continuously modified by the previous days' cell death, actual cell removal by phlebotomy, and the change in production pattern. For example, on day 1 the red cell age was assumed to be random so that 1/120th of the red cell mass died on that day. With removal of cells by phlebotomy or test sampling, the age distribution was skewed by the appearance of a younger population at increased levels of marrow production. Thus, on each subsequent day, the quantity of senescent cells was recalculated to adjust for cell loss and to exclude the increased production of young cells by use of the following formulas:

(footnote 3 continued)

$$\text{DED (day 1)} = 1/120 \times \text{RCV}$$

$$\text{RCV (day 2)} = \text{RCV (day 1)} - \text{DED (day 1)}$$

$$\text{DED (day 2)} = \frac{1}{119} \left[\text{RCV (day 2)} - \text{REM} \times \frac{\text{RCV (day 2)}}{\text{TBV} \times \text{Hct (day 2)} \times 0.91} \right]$$

$$\text{RCV (day 3)} = \text{RCV (day 2)} - \left[\text{DED (day 2)} + \text{REM} \times \frac{\text{RCV (day 2)}}{\text{TBV} \times \text{Hct} \times 0.91} \right]$$

$$\text{DED (day 3)} = \frac{1}{118} \left[\text{RCV (day 3)} - \text{REM} \times \frac{\text{RCV (day 3)}}{\text{TBV} \times \text{Hct (day 3)} \times 0.91} \right] \text{ etc.}$$

Therefore, for each period of constant anemia, total production was estimated as the sum of the amount of red cells removed plus the calculated amount of cells lost from senescence. Daily production was expressed in two ways: (a) as production/day by relating the total production to the configuration of the reticulocyte curve (Fig. 1) and (b) as mean daily production for accumulated periods of not less than 7 days during periods of constant reticulocytosis. The *production index* equaled the mean daily production divided by 1/120th of the original red cell volume (the assumed basal state production for the subject).

For reasonable accuracy, this calculation required that (a) all new cells produced during the study have a life-span of at least 60 days and (b) the mean corpuscular hemoglobin concentration of the new cells remains normal. The latter was monitored with frequent red cell constants during the studies. The first assumption was examined by comparing the final calculated life-span distribution to an actual determination of the ^{51}Cr life-span of the circulating red cells at the end of the phlebotomy study. 100 ml of whole blood was obtained on the 50-70th day of the phlebotomy study and labeled with 150 μC of ^{51}Cr in the presence of acid citrate dextrose (ACD) solution. After washing the cells with sterile saline, one-third of the original sample was injected into each of three normal recipients. Blood samples were obtained three times a week for the next 7 wk to determine the ^{51}Cr disappearance rate. Red cell life-spans were expressed both as the $t_{1/2}$ disappearance of ^{51}Cr and as the percentage of labeled red cells still circulating at 50 days after correction for a 1% ^{51}Cr elution rate.

Reticulocyte production. Reticulocyte counts were performed twice daily throughout the studies. After incubation with new Methylene blue, cover slip smears were counterstained, mounted in pairs, and at least 5000 consecutive red cells were enumerated for each time point by two observers. The observed reticulocyte counts were corrected for variations in hematocrit to obtain an absolute reticulocyte percentage:

Absolute reticulocyte count (%)

$$= \text{Observed reticulocyte count (\%)} \times \frac{\text{Observed Hct}}{45}$$

Marrow and reticulocyte maturation studies

Marrow iron transit times. The marrow iron transit time was measured after injection of ^{59}Fe for the plasma (footnote 3 concluded)

DED = Cell dying in next 24 hr.

RCV = Red cell volume (ml) as measured by ^{51}Cr -labeled red cell mass.

RCV (day 2, 3, etc.) = That portion of the red cell mass remaining after phlebotomy and the previous days' cell death.

REM = Red cells (ml) removed by phlebotomy.

TBV = Total blood volume (ml) as determined from the ^{51}Cr -labeled red cell mass and plasma volume measurements of the transferrin- ^{59}Fe clearances.

Hct = Hematocrit (%) microhematocrit method.

0.91 = Total body hematocrit: venous hematocrit ratio (15).

iron turnover determination. Whole blood was obtained twice daily for 10 days after injection and 2 ml counted for radioactivity. We measured the transit time (days) as the time from 50% clearance of radioiron from the plasma to 50% reappearance in red cells, assuming the 10-day level as 100% reappearance. Measurements were performed in the basal state and at each level of anemia once production had reached a plateau.

Reticulocyte maturation. Circulating reticulocyte maturation was characterized by (a) classification of reticulocyte content according to Heilmeyer (16) and (b) calculation of a circulating reticulocyte maturation time.

(a) In two subjects, daily reticulocyte smears were graded for individual cell reticulocyte content. At least 100 reticulocytes were examined on each smear and graded on a scale of 0-IV, where type 0 = a typical orthochromatic normoblast or a cell which contains both nuclear fragments and large amounts of reticulum, I = a macrocytic cell nearly filled with a large amount of reticulum, II = a macrocytic cell containing a number of strands of reticulum, III = a normocytic or slightly macrocytic cell with a few strands of reticulum, and IV = a normocytic cell with a single strand or a few fragments of reticulum.

(b) In view of the progressive reduction in transit time and increase in amount of reticulum in the reticulocytes of anemic individuals, the discrepancy between the absolute reticulocyte counts and other measurements of production was assumed to represent in large part a prolongation of the time required for reticulocyte to disappear from the circulating reticulocyte, the circulating reticulocyte maturation time. This assumption is supported by experiments in animal models in which in vivo studies of maturation of reticulum, ribosomes, and the ability to synthesize hemoglobin have been carried out (reference 18 and footnote 4). A circulating reticulocyte maturation time was therefore calculated at each hematocrit level from the absolute reticulocyte counts and the plasma iron turnover and (or) phlebotomy production measurements.

Circulating reticulocyte maturation time (days)

$$= \frac{\text{Absolute reticulocyte count (\%)}}{\text{Plasma iron turnover or phlebotomy production index}}$$

Normal maturation time (days) = 1.0.

Throughout all studies frequent measurements of red cell constants, serum iron, and total iron binding capacity (18, 19), smear morphology, and serum proteins were obtained.

RESULTS

Eight subjects were studied both in the normal state and after induction of specific levels of anemia by graded phlebotomy. The marrow production response was determined from serial measurements of the plasma iron turnover, reticulocyte counts, and the level of daily phlebotomy, as shown for one of the subjects (Fig. 1). This determination permitted a comparison of the three measurements of production as well as characterization of reticulocyte behavior with increasing anemia.

*Ganzoni, A., R. S. Hillman, and D. Heywood. Hemoglobin synthesis during in vivo maturation of the rat reticulocyte. Data to be published

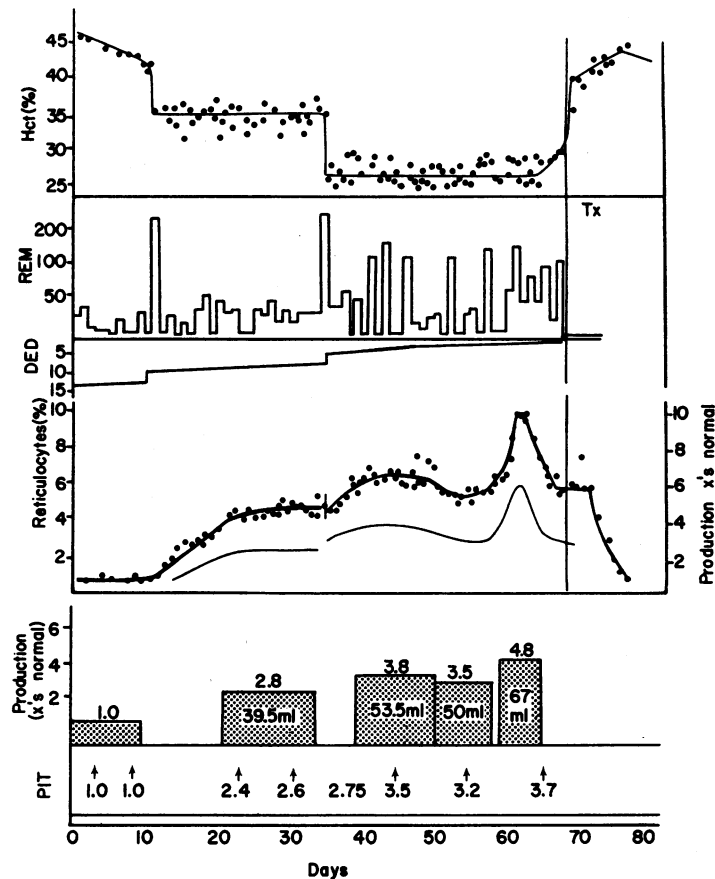


FIGURE 1 Serial measurements of marrow production in one subject during a control period and after the hematocrit was lowered to 32–37% and subsequently 25–30% for 3–5 wk. The hematocrit was kept constant by daily graded phlebotomy. (*REM* = amount of blood removed each day as packed red cells.) Cell senescence (*DED*) was calculated daily (see Methods) and added to the amount of cells removed to obtain a phlebotomy production measurement. This was expressed as mean daily production for periods of constant reticulocytosis (bar graphs indicating both milliliters of cells produced each day and production index) and by smoothing the total production for each hematocrit level to the shape of the reticulocyte curve (solid line under the reticulocyte curve). Measurements of plasma iron turnover were performed at 7- to 10-days intervals and are expressed as the PIT index. Reticulocyte counts (closed circles) were obtained twice daily and corrected for hematocrit variation.

Measurements of marrow production

Phlebotomy production measurement. As seen in Fig. 1, the amount of cells removed by phlebotomy (*REM*) was continuously adjusted as marrow production increased to maintain the hematocrit level first between 32 and 37% and subsequently between 25 and 30%. Daily levels of marrow production were then estimated from the amount of packed red cells removed by phlebotomy plus the estimated amount of cells lost from senescence (see Methods). To perform this calculation it was essential that all new red cells produced under

these anemic conditions have both a life-span greater than 60 days and a normal mean corpuscular hemoglobin concentration (MCHC). The first requirement was examined by performing a ^{51}Cr life-span determination on the cells at the end of the study (Fig. 2). After 65 days of continuous phlebotomy, the calculated red cell age distribution for the subject shown in Fig. 1 was markedly shifted to a younger aged population; 93% of the circulating cells were less than 60 days old. When this population was labeled with ^{51}Cr and their life-span determined in three normal recipients, Cr $t_{1/2}$ disappear-

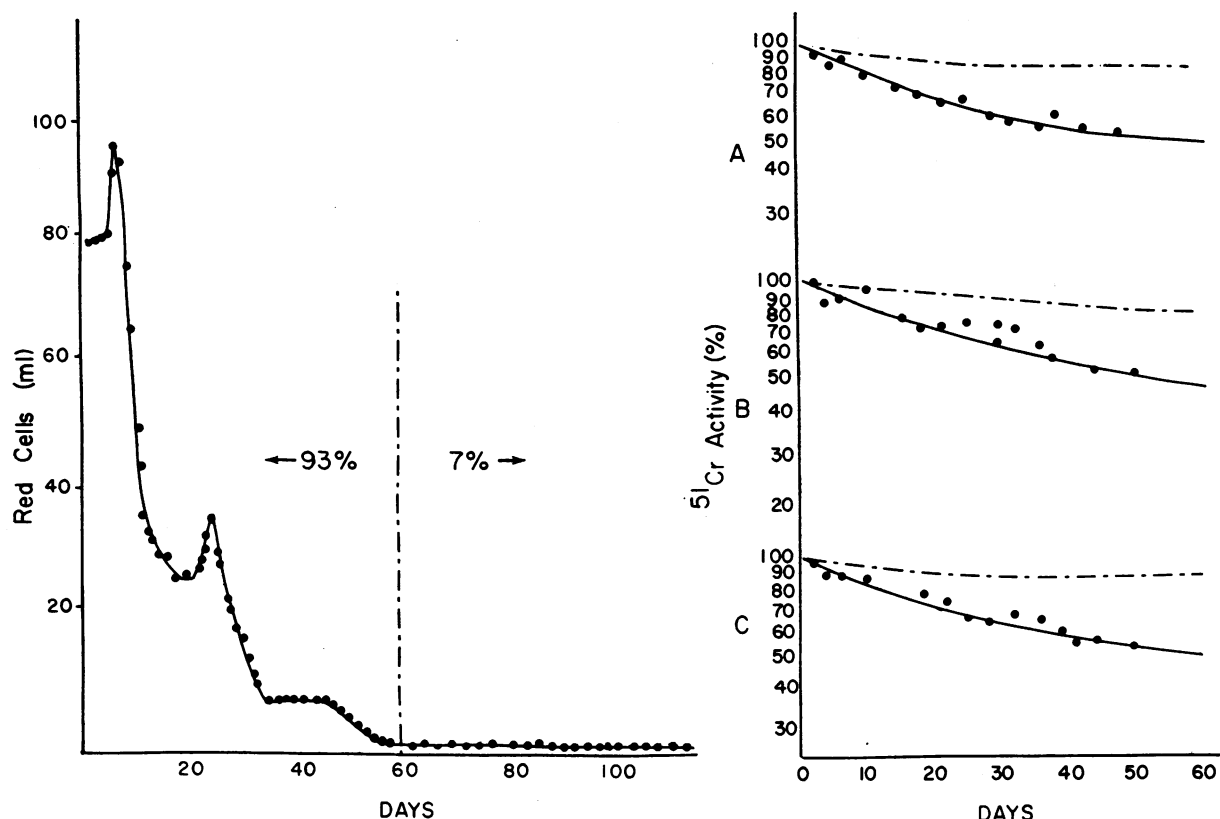


FIGURE 2 Red cell age distribution and red cell life-spans after 65 days of continuous phlebotomy for the subject depicted in Fig. 1. The red cell age distribution was calculated from the phlebotomy data and the combined measurements of marrow production with the assistance of a digital computer. If a uniform age distribution on day 1 is assumed, the change in age distribution was calculated on each successive day of phlebotomy taking into account cell loss from senescence and phlebotomy and increased cell entry by way of increased red cell production. By the 65th day, it was calculated that 93% of the circulating red cell population was less than 60 days of age. When these cells were labeled with ^{51}Cr , $t_{1/2}$ disappearance of 50, 55, and 57 days were observed after administration to three normal recipients. After correction for a 1% ^{51}Cr elution rate (broken lines), 85–90% of the original labeled cells were still circulating in these recipients after 50 days. This confirmed the fact that the predicted shift to a younger population had occurred and that these cells had a relatively normal life-span.

ances of 50, 55, and 57 days were observed. If a 1% ^{51}Cr elution rate is assumed, this indicated that 85–90% of the labeled cell population was still circulating at 50 days, a fact confirming the viability of the anemia-induced erythrocytes. Similar results for three other subjects are shown in Table I. As for the second requirement, serial measurements of mean-cell constants were performed during the studies (Fig. 3). Although the mean corpuscular volume (MCV) and mean corpuscular hemoglobin content (MCH) rose slightly at hematocrit 25–30%, the MCHC did remain constant. Both findings supported the validity of the phlebotomy production measurement. This was further supported by the excellent correlation with the plasma iron turnover measurements (see below).

Plasma iron turnover measurements. Over a wide range of marrow production levels, one to five times

normal, the plasma iron turnover indices were in close agreement with the phlebotomy production measurements (Fig. 4). On only four occasions was there a major discrepancy between the two measurements. In each instance, the plasma iron turnover was performed at a time when the serum iron values were less than 50 $\mu\text{g}/100\text{ ml}$.

Reticulocyte iron uptake was studied in conjunction with the measurements of plasma iron turnover (Table II). At higher levels of marrow production, when the reticulocyte counts were 13.0–15.0%, in vitro red cell uptake increased to a maximum of 0.52 $\mu\text{g}/\text{ml}$ red cells after a 120 min incubation. If it is assumed that in vivo reticulocyte uptake would be of a similar magnitude, it may be estimated that at the highest levels of marrow production with plasma iron turnovers of 8–10,000 μg every 2 hr less than 500 μg was directly incorporated

TABLE I
*⁵¹Cr Life-Spans of Cell Populations after 50–65 days
of Continuous Phlebotomy*

Patient	⁵¹ Cr $t_{1/2}$ disappearance	Labeled cells re- maining at 50 days*	Calculated age dis- tribution of cells less than 60 days age
		%	%
A	50	85	93
	55	88	
	57	90	
B	53	86	91
	60	92	
	15	—	
C	45	79	86
	55	84	
D	52	83	89
	56	85	

* Assuming 1% elution of ⁵¹Cr.

into circulating reticulocytes. The primary distribution of injected radioiron continued to be nucleated marrow precursors.

Reticulocyte production measurements. With anemia, the absolute reticulocyte counts (the observed reticulocyte counts corrected for hematocrit variations) were consistently higher than actual marrow production (Figs. 1 and 5). This discrepancy was not a function of the level of marrow production; the absolute reticulocyte counts exceeded the plasma iron turnover or phlebotomy balance indices by a factor of 1.5–3.0 both when production was two to three times normal and at levels of five times normal (Fig. 5).

Marrow and reticulocyte maturation studies

Measurements of the marrow iron transit time were made at each hematocrit level (Fig. 6). A progressive shortening of the iron transit time occurred as the severity of the anemia increased. By the time the hematocrit was reduced to 25–30% the marrow iron transit time had shortened to 1.5 days as compared to a normal time of 3.5 days. This was associated with the appearance in circulation of large, polychromatic reticulocytes containing greater than normal amounts of reticulum, marrow reticulocytes. When new Methylene blue-stained reticulocyte smears were graded according to the Heilmeyer classification (16) it was apparent that the number of cells containing increased amounts of reticulum correlated with the hematocrit level (Fig. 7). Whereas normal subjects demonstrated 75% grade IV and only

25% grade III reticulocytes, the percentage of grade III reticulocytes increased to 40% at hematocrit of 35 and 60–70% at hematocrit of 25–27%. At the lower hematocrit level, approximately 5–10% of grade II reticulocytes were also seen.

Both findings imply a premature delivery into circulation of marrow reticulocytes which require a longer than normal period of maturation in circulation to lose their reticulum. Since this could in large part explain the discrepancy between the reticulocyte counts and production indices, approximate circulating reticulocyte maturation times were calculated from the absolute reticulocyte counts and plasma iron turnover production indices at varying levels of marrow production and hematocrit depression. As shown in Fig. 8, when these reticulocyte maturation times were compared to the hematocrit level, they progressively lengthened with increasing anemia. From a normal value of 0.8–1.2 days,

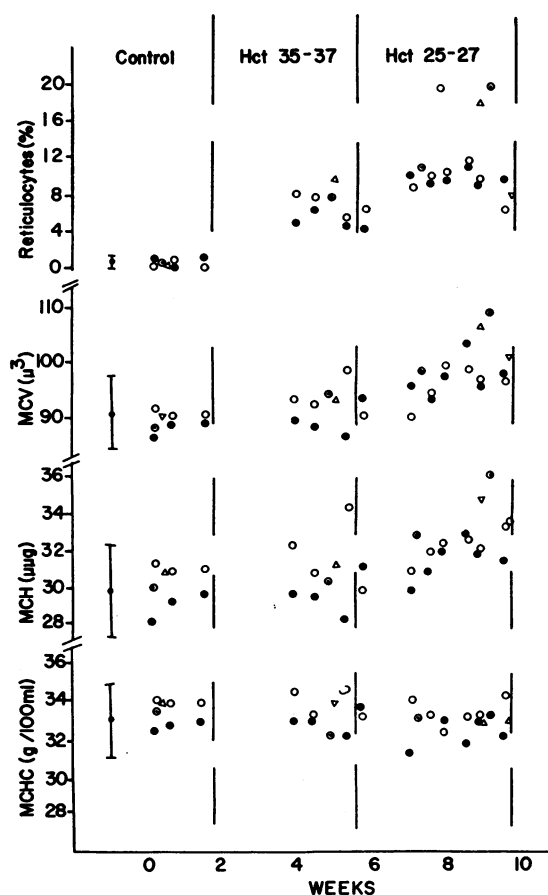


FIGURE 3 Mean cell constants of four subjects during phlebotomy to hematocrits of 32–37% and 25–30%. The mean corpuscular volume (MCV) and mean corpuscular hemoglobin content (MCH) increased slightly, while the mean corpuscular hemoglobin concentration (MCHC) remained constant throughout the studies.

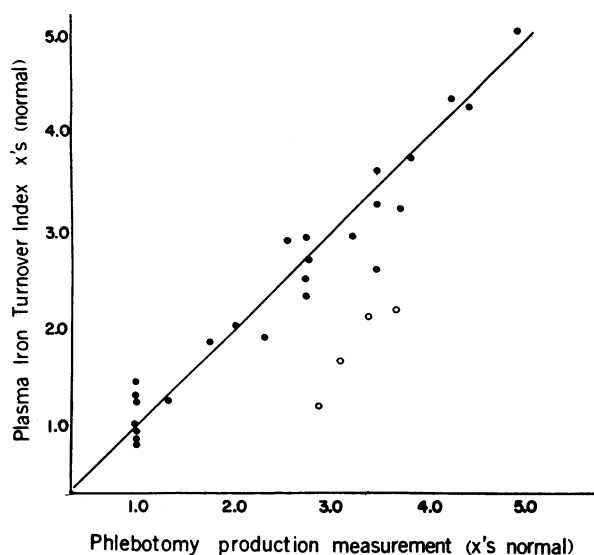


FIGURE 4 A comparison of the plasma iron turnover indices with the phlebotomy production measurement. When measurements of the plasma iron turnover were performed at a time when the serum iron was less than $50 \mu\text{g}/100 \text{ ml}$ (open circles), the plasma iron turnover gave a falsely low measure of production.

the circulating reticulocyte maturation time lengthened to 1.5–2 days at hematocrit of 32–37% and 1.7–3 days at hematocrit of 25–30%.

The prolongations of the maturation time of the circulating reticulocyte were also compared to the measurements of marrow iron transit time. If the reticulocyte maturation prolongation were merely a reflection of premature delivery to circulation of otherwise normal reticulocytes, a comparable shortening of the marrow iron transit time might be expected for increasing prolongations of circulating reticulocytes maturation. As shown in Fig. 9, when the transit time shortened from 3.5 to 2.5 days there was a comparable prolongation of reticulocyte maturation. However, as the transit time fell to below 2 days the circulating reticulocyte maturation time in most subjects remained between 1.5 and 2

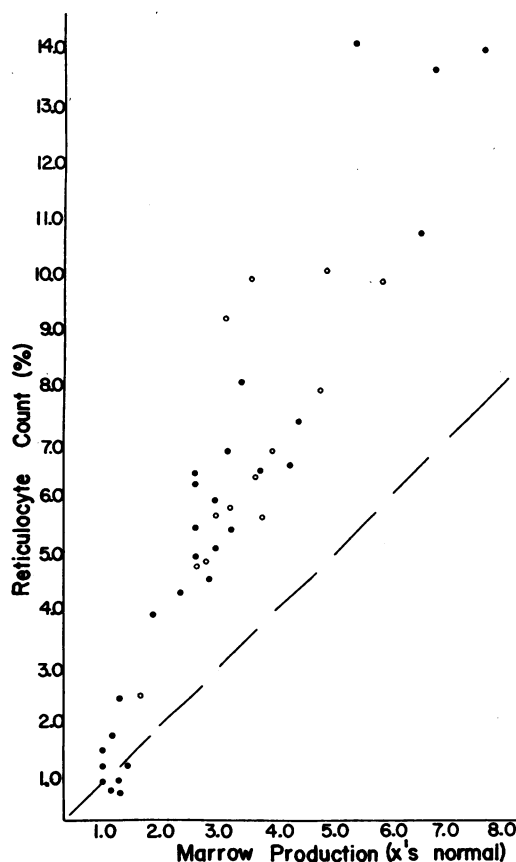


FIGURE 5 A comparison of the absolute reticulocyte counts and plasma iron turnover (closed circles) or phlebotomy production indices (open circles) demonstrated a poor correlation. The absolute reticulocyte count exceeded marrow production by one and one-half to three times normal over a wide range of production levels.

days. In only two subjects, the patients with hemochromatosis, did a comparable prolongation of the circulating reticulocyte maturation time occur for the lower values of marrow iron transit time.

DISCUSSION

Whereas past studies of the human erythroid marrow's response to anemia have been largely limited to pathological states (1–5), the present measurements of marrow production were carried out in normal individuals during prolonged periods of phlebotomy-induced anemia. The results would support the previous conclusion that the plasma iron turnover reflects the level of erythroid marrow activity (6–10, 13). Over a wide range of marrow production levels, there was excellent correlation between the plasma iron turnovers and the marrow production level as estimated from the level of daily phlebotomy required to maintain the hematocrit at a

TABLE II
Reticulocyte Uptake of Radioiron

Reticulocyte count	Number of determinations	Iron uptake	
		60 min	120 min
%		$\mu\text{g}/\text{ml red cells}$	
0.5–1.5	3	0	0–0.12
3.5–5.0	5	0–0.05	0–0.21
5.0–10.0	4	0.15–0.30	0.15–0.50
10.0–15.0	6	0.05–0.42	0.18–0.52

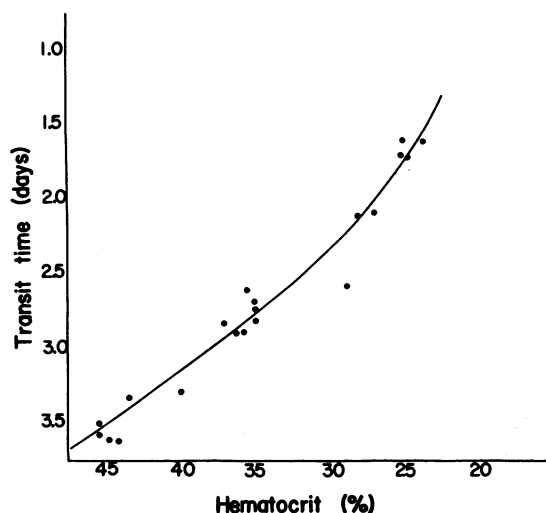


FIGURE 6 Marrow iron transit times were measured at hematocrit levels ranging from 45 to 25%. As the hematocrit was lowered the transit time shortened from 3.5 to 1.7 days.

constant level. Only when the serum iron fell below 50 $\mu\text{g}/100\text{ ml}$ did the measurements of plasma iron turnover fail to accurately reflect marrow production. In these situations, production apparently continued at

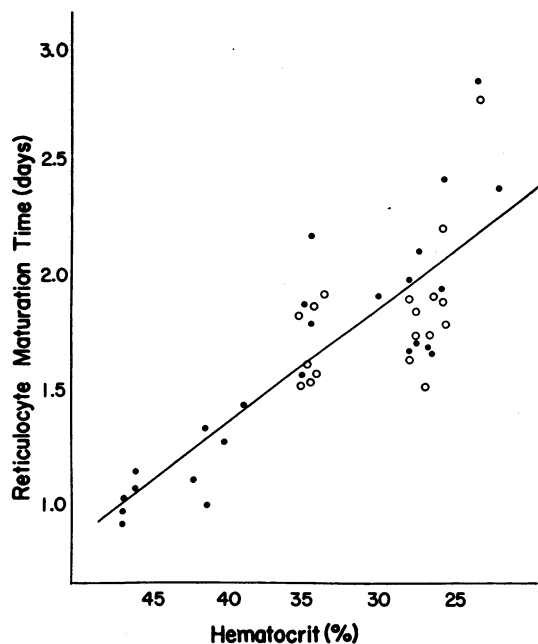


FIGURE 8 Progressive prolongation of the circulating reticulocyte maturation time occurred with depression of the hematocrit. If we assume that the discrepancy between the absolute reticulocyte count and plasma iron turnover is primarily related to a premature delivery of marrow reticulocytes which then require a longer time to lose their reticulum, the reticulocyte maturation time was calculated as:

$$\text{Reticulocyte maturation time (days)} = \frac{\text{Absolute reticulocyte count}}{\text{Production index}}$$

Closed circles, calculated from the plasma iron turnover; Open circles, calculated from phlebotomy production measurements.

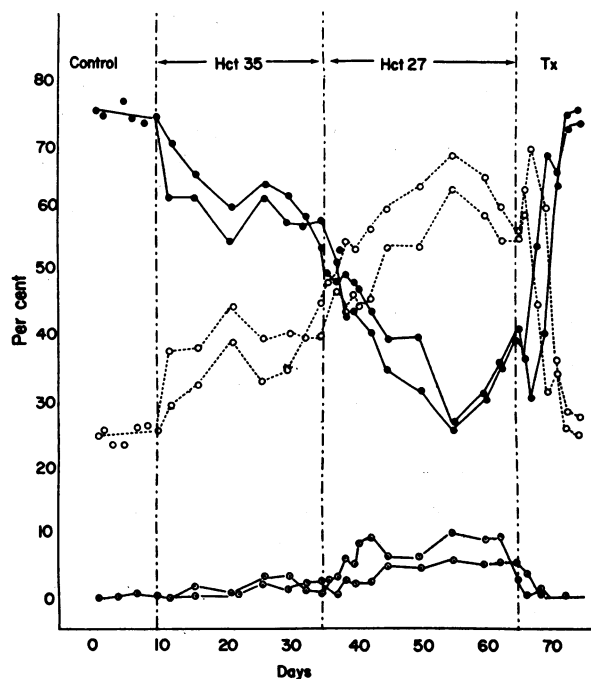


FIGURE 7 Reticulocytes were graded for their relative content of reticulum according to the Heilmeyer classification (grade IV, closed circles; grade III, open circles; grade II, circled dots). As the hematocrit was lowered large numbers of reticulocytes containing increased amounts of reticulum, grade III and II types, appeared.

levels of two to three times normal at a time when measurements of plasma iron turnover were less than twice normal. In part, this may reflect the fact that the plasma iron turnover only measures iron delivery to the marrow at one point in time and does not necessarily indicate total iron supply over a 24 hr period. However, the discrepancy may also imply a mechanism of direct transfer of iron from reticuloendothelial cells to marrow precursors, which becomes important at lower serum iron values.

When the absolute reticulocyte counts were compared to the measurement of marrow production by plasma iron turnover, the reticulocyte counts were consistently higher than actual marrow production over a wide range of production levels (Fig. 5). At the same time, large numbers of polychromatic macroreticulocytes appeared in circulation. Both the number of these cells and their relative reticulum content progressively increased as the hematocrit was lowered. Reiff, Nutter, Donohue, and

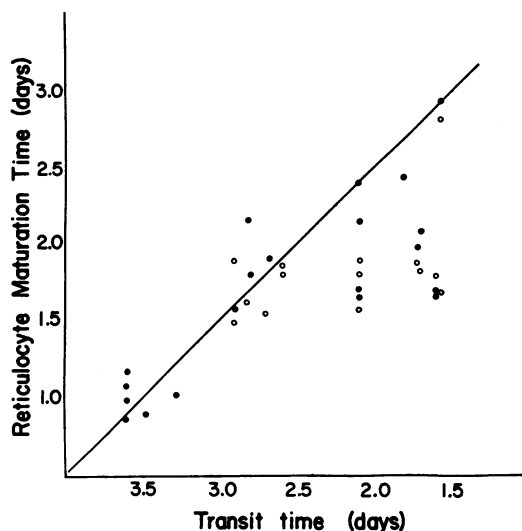


FIGURE 9 Prolongation of the circulating reticulocyte maturation time (closed circles, calculated from plasma iron turnovers; open circles, calculated from phlebotomy production measurement) is compared to the shortening of the marrow iron transit time. At a hematocrit of 25 the transit time reduction was not accompanied by an equal prolongation of the reticulocyte maturation time in the normal subjects. Only the patients with hemochromatosis demonstrated a comparable prolongation.

Finch (20) and Gordon, LoBue, Dornfest, and Cooper (21) have shown in the rabbit and rat that marrow reticulocytes are released from the marrow prematurely in response to anemia or erythropoietin stimulation. Ganzoni, Hillman, and Finch (17) have further demonstrated that these rat macroreticulocytes have a prolonged maturation time *in vivo*. This is accompanied by reduction in the marrow iron transit time, an approximate measurement of the time required for basophilic normoblasts to mature to marrow reticulocytes and be delivered into circulation. Similarly, in man, the appearance of increasing numbers of macroreticulocytes containing greater than normal amounts of reticulum is associated with a progressive shortening of the iron transit time. This would suggest that in large part the discrepancy between the absolute reticulocyte count and actual marrow production levels is the result of a prolongation of the circulating reticulocyte maturation time. Since prematurely delivered marrow reticulocytes require an extra 1 or 2 days to lose their increased amounts of reticulum, they tend to accumulate in circulation and falsely elevate the reticulocyte count.

Before the concept of a prolongation of the circulating reticulocyte maturation time is accepted as the lone cause of the discrepancy between the absolute reticulocyte counts and other measurements of marrow production, it is important to exclude other disturbances in

reticulocyte maturation. In small animals, Ambs (22) and Brecher and Stohlman (23) have demonstrated the occurrence of skipped mitoses at the basophilic or polychromatic normoblast stage which results in the repopulation of the circulation with macrocytes containing greater than normal amounts of hemoglobin. Since such cells may be short-lived (24–26), this phenomenon is of potential importance in the interpretation of the reticulocyte count, both in terms of prediction of the maturation time of the circulating reticulocyte and the correlation of the reticulocyte count with total marrow production. However, Ganzoni, Hillman, and Finch (17) have recently shown that these cells do circulate for at least one-third of their normal life-span so as not to interfere with a full expression of the phenomenon of reticulocyte maturation time prolongation. Moreover, in normal man, there is little evidence for a significant reduction in the number of mitoses within the marrow. The increases in the MCV and MCH in the present studies were not of a magnitude which would implicate skipped mitosis as an important mechanism in man during periods of moderate anemia. Furthermore, when life-spans of the phlebotomy-induced red cell populations were determined, a significant number of short-lived cells was not observed. Of course, this measurement would not have detected a loss of cells with a life-span of only a few days. However, this type of wastage seems unlikely. Not only have studies of small animals failed to demonstrate an appreciable loss of macroreticulocytes within the first few days (17), but also any major loss of short-lived cells in the present studies should have been reflected by a major discrepancy between the plasma iron turnover and the phlebotomy production measurement. For these reasons, the present studies would indicate that in man, at least, the high levels of marrow production and changes in marrow maturation which are induced by moderate anemia do not result in an abnormal reticulocyte product in which changes in reticulocyte maturation or life-span will invalidate the use of the reticulocyte count as a measurement of effective marrow production.

The prolongation of the circulating reticulocyte maturation time reflects the severity of the anemia. When circulating reticulocyte maturation times were calculated from the absolute reticulocyte counts and plasma iron turnover measurements, progressive prolongation of the maturation time occurred as the severity of the anemia increased (Fig. 8). This was accompanied by a shortening of the marrow iron transit time. At a hematocrit of 32–37%, the transit time shortened commensurate with the prolongation of the reticulocyte maturation so that the total maturation time, the sum of the two measurements, remained constant. At the 25–30% hematocrit level, further shortening of the marrow iron transit

time was not necessarily reflected in equal prolongation of the reticulocyte maturation. In this regard, it should be noted that there was a concomitant prolongation of reticulocyte maturation for the degree of iron transit time reduction in both patients with hemochromatosis. However, in those individuals relying on orally administered iron or reticuloendothelial iron stores, the total maturation period fell from a normal value of 4.3–4.5 to 3.4–4.0 days. In view of the limitation of iron supply in the latter subjects, relative iron deficiency may have caused either a reduction in the total reticulocyte maturation period or a change in the distribution of radio-iron to marrow precursors so as to inordinately shorten the transit time. This theory is supported by previous studies of pernicious anemia patients during vitamin B₁₂ therapy (27), in which the iron supply was well maintained during studies of their marrow maturation characteristics. In this situation, measurements of the marrow iron transit time and reticulocyte maturation time at hematocrit level of 15% demonstrated a comparable shortening of the transit time for a prolongation of reticulocyte maturation of 2.3–2.5 days.

The concept of a progressive prolongation of the circulating reticulocyte maturation time with increasing levels of erythropoietin stimulation is an important consideration in interpreting the reticulocyte response in pathological states. Only by correcting the absolute reticulocyte count for the appropriate prolongation of circulating reticulocyte maturation can a reasonable index of production be obtained. Although it is presently impossible to express this prolongation as a function of measured erythropoietin levels, it is possible to estimate a maturation time for a specific level of hematocrit depression in those states in which erythropoietin stimulation appears appropriate for the severity of the anemia (28). Thus, as the hematocrit is reduced to approximately 35%, the maturation time of the circulating reticulocyte increases from 1.0 to 1.5–1.7 days. With more severe anemia the maturation time will increase to 1.7–2.0 days at a hematocrit of 20–30% and to as much as 2.3–2.5 days at a hematocrit below 20% (27). This estimated maturation time may then be employed to calculate a *corrected* reticulocyte count and production index by using the formula:

Corrected, absolute reticulocyte count (%)

$$= \frac{\text{Absolute reticulocyte count}}{\text{maturation time (days)}}$$

Reticulocyte production index

$$= \frac{\text{Corrected reticulocyte count}}{1.0}$$

⁵ The normal reticulocyte count and reticulocyte index is 1.0 (27).

ACKNOWLEDGMENTS

I gratefully acknowledge the assistance of Doctors Peter Ways and Clement Finch for their interest and advice and Dr. Paul Worrell, Mrs. Maura Oakes, Mrs. Mudite Petersons, and Mrs. Caryl Finholt for their technical help.

This work was supported in part by U. S. Public Health Service Grant AM 09950. A portion of the work was conducted through the University of Washington Clinical Research Center at King County Hospital, supported by the National Institute of Health (Grant FR-133).

REFERENCES

- Giblett, E. R., D. H. Coleman, G. Pirzio-Biroli, D. M. Donohue, A. G. Motulsky, and C. A. Finch. 1956. Erythrokinetics. Quantitative measurements of red cell production and destruction in normal subjects with anemia. *Blood*. **11**: 291.
- Huff, R. L., T. G. Hennessy, R. E. Austin, J. F. Garcia, B. M. Roberts, and J. H. Lawrence. 1950. Plasma and red cell iron turnover in normal subjects and in patients having various hematopoietic disorders. *J. Clin. Invest.* **29**: 1041.
- Huff, R. L., P. J. Elmlinger, J. F. Garcia, J. M. Oda, M. C. Cockrell, and J. H. Lawrence. 1951. Ferrokinetics in normal persons and in patients having various erythropoietic disorders. *J. Clin. Invest.* **30**: 1512.
- Finch, C. A., D. H. Coleman, A. G. Motulsky, D. M. Donohue, and R. H. Reiff. 1956. Erythrokinetics in pernicious anemia. *Blood*. **11**: 807.
- Sturgeon, P., and C. A. Finch. 1956. Erythrokinetics in Cooley's anemia. *Blood* **12**: 64.
- Bothwell, T. H., A. V. Hurtado, D. M. Donohue, and C. A. Finch. 1957. Erythrokinetics. IV. The plasma iron turnover as a measure of erythropoiesis. *Blood*. **12**: 409.
- Lawrence, J. H., R. L. Huff, W. Siri, L. R. Wasserman, and T. G. Hennessy. 1952. A physiological study in the Peruvian Andes. *Acta Med. Scand.* **142**: 117.
- Reynarfarje, C., R. Lozano, and J. Valdivieso. 1959. The polycythemia of high altitudes: iron metabolism and related aspects. *Blood*. **14**: 433.
- Lawrence, J. H., P. J. Elmlinger, and G. Fulton. 1952. Oxygen and the control of red cell production in primary and secondary polycythemia. Effects on the iron turnover patterns with Fe⁵⁹ as tracer. *Cardiologia*. **21**: 337.
- Pollycover, M., and R. Mortimer. 1961. The quantitative determination of iron kinetics and hemoglobin synthesis in human subjects. *J. Clin. Invest.* **40**: 753.
- Cline, M. J., and N. I. Berlin. 1963. The reticulocyte count as an indicator of the rate of erythropoiesis. *Amer. J. Clin. Pathol.* **2**: 121.
- Crosby, W. H., C. A. Finch, F. I. Haurain, F. Stohman, and L. Wasserman. 1961. Modern methods for quantitation of red blood cell production and destruction: erythrokinetics. *Blood*. **18**: 225.
- Hillman, R. S., and P. Henderson. 1969. The control of marrow production by the level of iron supply. *J. Clin. Invest.* **48**: 000.
- Bothwell, T. H., and C. A. Finch. 1962. Iron Metabolism. Little, Brown and Company, Boston.
- Mollison, P. L. 1967. Blood Transfusion in Clinical Medicine. F. A. Davis Co., Philadelphia.
- Heilmeyer, L. 1931. Blutfarbstoff wech sel studien. *Deut. Arch. Klin. Med.* **171**: 121.

17. Ganzoni, A., R. S. Hillman, and C. A. Finch. 1969. Maturation of the macroreticulocyte. *Brit. J. Haematol.* **16**: 119.
18. Bothwell, T. H., and B. Mallett. 1955. The determination of iron in plasma or serum. *Biochem. J.* **59**: 599.
19. Morgan, E. H., and G. Carter. 1960. Plasma iron and iron binding capacity levels in health and disease: with an improved method for estimation of plasma iron concentration and total iron binding capacity. *Australas. Ann. Med.* **9**: 209.
20. Reiff, R. H., J. Y. Nutter, D. M. Donohue, and C. A. Finch. 1958. The relative number of marrow reticulocytes. *Amer. J. Clin. Pathol.* **30**: 199.
21. Gordon, A. S., J. LoBue, B. S. Dornfest, and G. W. Cooper. 1962. Reticulocyte and leukocyte release from isolated perfused rat legs and femurs. In *Erythropoiesis*. Grune & Stratton, Inc., New York. 321.
22. Ambs, E. 1960. Über Grosse Und ham oglobinreiche reticulocytin inkerkrise von aneinien. *Schweiz. Med. Wochenschr.* **90**: 413.
23. Brecher, G., and F. Stohlman, Jr. 1961. Reticulocyte size and erythropoietic stimulation. *Proc. Soc. Exp. Biol. Med.* **107**: 887.
24. Stohlman, F., Jr. 1961. Humoral regulation of erythropoiesis. VII. Shortened survival of erythrocytes produced by erythropoietin or severe anemia. *Proc. Soc. Exp. Biol. Med.* **107**: 884.
25. Berlin, N. J., and C. Lotz. 1951. Lifespan of the red blood cell of the rat following acute hemorrhage. *Proc. Soc. Exp. Biol. Med.* **78**: 788.
26. Card, R. T., and L. S. Valberg. 1967. Characteristics of shortened survival of stress erythrocytes in the rabbit. *Amer. J. Physiol.* **213**: 566.
27. Hillman, R. S., J. Adamson, and E. R. Burka. 1968. Characteristics of the vitamin B₁₂ correction of the abnormal erythropoiesis of pernicious anemia. *Blood.* **21**: 419.
28. Hillman, R. S., and C. A. Finch. 1967. Erythropoiesis, normal and abnormal. *Seminars Hematol.* **4**: 327.