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Contents

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ABSTRACT

The course of development of red blood cells in the rat was studied by use of a radioiron label; determinations of Fe^{59} indicated changes in the blood. The following findings apply to male rats maintained on a standard laboratory diet.

The unit blood volume decreases continuously as a function of age reaching a constant level at approximately 200 days of age.

The unit plasma volume increases slightly from birth to 15 days of age, and then decreases to an adult level after 200 days.

The unit red cell volume and unit hemoglobin decrease from birth to low levels between 15 and 20 days of age, returning to a constant level by 60 days of age. There is also a significant decrease in unit red cell volume after 160 days of age.

Throughout the anemic period there is a continual increase in the total red cell volume, and in fact, the daily gain in red blood cells per gram of rat is greater during this period than at any time thereafter. It is thus concluded that the anemia observed is not due to a lack of erythropoietic stimulation but is simply a reflection of the rapid growth rate in these young rats and the inability of the erythropoietic tissue to keep up with this growth rate.

Hypoxia was shown to be ineffective as an erythropoietic stimulus in young rats throughout the anemic period. It was also shown that the relative daily gain in red blood cells in adule rats in response to a severe hypoxic stimulus does not exceed the relative daily gain in red blood cells existing in young rats in response to growth. This leads to the conclusion that the high rate of red cell production existing in young rats throughout the anemic period is maximal or near maximal.

Radioiron turnover studies indicate that per unit body weight, the 15-day-old rat has a red cell iron replacement rate approximately three times that existing in the adult rat. Also it was seen that the spleen of the 15-day-old rat produces approximately 30% of the red cells in this animal.

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INTRODUCTION

Many studies have been made of the variations in the blood picture as a function of age in the normal rat, as well as in other species. Jolly,[66] in 1909, observed a decrease in the hemoglobin concentration in the rat from birth to a low level at between 8 and 14 days of age, after which it rose slowly to normal values at between 6 and 8 weeks. Many other investigations have substantiated this finding in the rat,[14, 29, 30, 68, 121] as well as in other species.[75] Such a fall in hemoglobin concentration following birth has been observed in man, with low levels reached at 2 to 3 months and returning to normal by 6 to 12 months.[1, 70, 71, 72, 119]

In the rat, in a few studies in which hematocrit determinations were made, it was observed that the hematocrit dropped to a low level at between 15 and 20 days and then returned to normal values by 50 days of age.[14,121]

Studies made in the rat of the variations in red cell count as a function of age have in general agreed that at birth the red cell count is low and that it slowly rises to normal values at around 50 days of age.[14,29, 81, 105, 121] This observation has also been made in other species;[75,120] however, in man the red cell count is essentially normal at birth.[120]

In the rat the red cell size is largest at birth and slowly decreases to normal values at approximately 40 days of age.[105, 121] Other species have also been observed to have red cell sizes larger than normal at birth.[72, 120] The concentration of hemoglobin within the red cells does not change in the rat.[121]

The reticulocyte percentage in the rat has been studied as a function of age by various authors.[14, 29, 54, 91, 105] In general the reticulocyte percentage has been very high at birth and for a few days thereafter. In some cases more than 90% of the red cells at birth were reticulocytes. This drops rapidly by the third or fourth day to a level of approximately 25% of the red cells. This still relatively high percentage of reticulocytes remains out to about 20 to 25 days of age, and then drops slowly to an adult level of 1% to 2%. In man also the reticulocyte percentage has been observed to be high at birth;[72, 76, 82] however, it usually reaches normal adult values by 10 days of age.

The bone-marrow picture in the rat at various ages has received some attention. Jolly noted in the rat that along with the anemia he observed a coincident abundance of mitoses and nucleated red cells in the marrow.[66] Kindred has made quantitative studies of the hemopoietic organs in rats of 15 and 80 days of age.[79, 80] He found extensive erythropoiesis occurring in the spleen of the rat at 15 days of age and some erythropoiesis in the spleen even at 80 days of age. He estimated that 22% of the red cells of the 15-day-old rat are produced in the spleen.

That red bone marrow in man fills the cavity of all bones in early childhood is well substantiated.[25, 65, 118] With progressing age the red marrow gradually recedes from the distal portions of the skeleton toward the trunk and is replaced by yellow marrow. It is also established that extramedullary hematopoiesis is common in the anemia of infancy.[13, 41, 88] This is regarded as a compensatory reaction due to an insufficient red cell production by the marrow.[67] Sabin observed in the rabbit that the proportion of erythroid to myeloid cells in the bone marrow is the reverse of that in the adult.[100]

The possibility of iron deficiency as a cause of the anemia of the newborn remains confusing. Bunge found that the total iron content of several mammalian species was highest at birth and that it diminished progressively during the suckling period.[15] Huggett observed that the 21-day-old rat has three times the amount of iron he is born with; in spite of this, relative to body weight the total body iron falls to half its birth level at 21 days.[64] This is the result of the very rapid increase in body weight relative to the increase in total body iron. Others also considered rapid growth as an important factor in the development of an iron-deficiency anemia.[55, 68] Usher obtained some evidence of repair of the neonatal anemia in man with iron.[111] Other investigators, however are not entirely convinced that relative iron deficiency is an important factor in the development of anemia of the newborn.[2, 22, 70, 72, 73] Josephs has applied the term "physiological anemia" to the anemia of the newborn and maintains that it is not influenced by any extrinsic factor.[71, 72] Recently, Horan has been unable to repair this anemia by iron administration.[58] Likewise, Contopoulos et al., working on rats, were unable to repair this anemia by the use of iron therapy.[22]

The possibility of a reduced erythropoietic stimulation has been considered as a cause for the anemia of the newborn by several authors.[22, 69] Contopoulos et al. report prevention of the neonatal anemia in rats by injections of erythropoietically active pituitary fractions.[22] Grant reports increased total body hemoglobin in rats and mice that were nursed by mothers placed in an intermittent hypoxic environment.[43]

Josephs more recently suggests the possibility that during the "physiological anemia" the hemopoietic tissues may be unable to meet the extra demands made upon them at this time owing to rapid growth.[74]

Wintrobe and Shumacker brought attention to the comparison that the increase in the number of red cells and the decrease in red cell size which take place in cases of pernicious anemia under the influence of liver therapy

are similar to the changes in the blood picture in the fetus and newborn in its normal development.[120, 121] They suggest that the anti-pernicious-anemia principle passes to the fetus from stores in the mother and that a relative deficiency of it is responsible for the anemia of the newborn. However, Bruner et al., after essentially confirming this comparison, point out that a dissimilarity is observed in the behavior of the reticulocytes, which remain at a high level throughout the anemic period in the rat.[14]

To summarize, in the rat an anemia is observed, as judged by hematocrit and hemoglobin concentrations, at about 15 days of age. Throughout this period a relatively high reticulocyte percentage is maintained along with evidence of a high erythropoietic state existing in the marrow as well as in the spleen.

The study reported herein was directed to the possible reasons for this apparently paradoxical situation, as well as to the general erythropoietic changes in the rat as a function of age. For simplicity the work was divided into three general phases. First, a study was made of the changes in total red cell volume as a function of age. Second, an attempt was made to determine the capability of rats of varying ages to respond to erythropoietic stimulation. Finally radioiron time-distribution studies were carried out in rats of various ages.

STUDIES OF THE CHANGES IN BLOOD, PLASMA, AND RED CELL VOLUME AS A FUNCTION OF AGE

With the advent of rapid and reliable blood volume techniques, more reports are dealing with the total quantities of the various constituents in the blood and less stress is being placed on studies involving only the concentration of the various blood constituents, which are subject to possible errors due to hemoconcentration or hemodilution. There are many good reviews of the various techniques of blood volume measurement.[31, 49, 56, 95]

The earliest techniques involved the direct measurement of the blood volume by collection of all the blood in an animal. Indirect methods involving the dilution of a substance that remains within the circulation have found more common usage. These methods can involve the labeling of either the plasma or the red cells.

Keith et al., in 1915, first introduced the dilution of a dye for the measurement of the blood volume.[78] Dawson et al., after trying many dye materials, found T-1824 the most suitable dye for blood-volume measurement.[27] However, this substance is a plasma diluent and perhaps should be limited in use to plasma-volume measurement. Radioiodine-labeled plasma has also been used for measurement of the plasma volume.[40] Sears et al. obtain close agreement for plasma-volume values by simultaneous employment of these two substances.[102]

The red cell volume has been measured by the use of various red cell labels. Among these are carbon monoxide,[20, 98] radioiron,[11] radiophosphorus,[5] radiopotassium,[57] and radiochromium.[46] In general, good agreement is obtained for red cell volume with these red cell labels, with the exception of carbon monoxide, which gives slightly higher values. Recently, Root et al., in a comparison of the carbon monoxide and radiophosphorus techniques, obtained values 12% higher with carbon monoxide than with radiophosphorus.[99] However, they point to the possibility that this difference may be a useful one, in that it may indicate the quantity of the erythropoietic tissue.

Studies in which blood volumes were determined at various ages have concluded in general that younger rats have larger blood volumes relative to body weight.[19, 21, 50, 85, 115] Metcoff and Favour, however, find the largest blood volumes relative to body weight in rats of 70 to 90 days of age.[87] The blood volume relative to body weight in man is largest in young infants and decreases with age.[86] Courtice[24] finds a linear relation between body weight and blood volume in various animal species, as do Morse et al.[90] in man.

Methods

The rats used in this study were males of the Long-Evans strain, fed on a complete laboratory diet.* All litters were reduced to six animals one day after birth, and the animals were weaned at 21 days of age. Since it was pertinent to the study, a growth curve was determined by periodic weighing of all the male rats in the stock colony at the Donner Laboratory from the date of birth to 360 days of age. The growth curve, presented in Fig. 1, is the result of more than 3000 observations, 1100 of which were weights of rats under 50 days of age.

The blood-volume determinations were made by use of the Fe^{59}-tagged-cell technique. At least 5 days prior to the blood-volume measurements, the blood of a donor rat was labeled by injecting the rat intraperitoneally with approximately 10 microcuries of Fe^{59} as ferric chloride. As will be seen later in the study, after an injection of Fe^{59}, the activity in the red blood cells reaches a peak at approximately 3 days and then plateaus. A sample of labeled blood from this donor was drawn in a heparinized syringe by a cardiac puncture. A portion of the donor blood was used for an hematocrit reading and the remainder was put into a serum vial to be used for the blood-volume determinations. Each determination was made with 0.05 to 0.2 milliliter of this donor blood, which contained from 0.01 to 0.1 microcurie of Fe^{59} radioactivity. The blood-volume determinations were all done under ether anesthesia.

The injection of tagged blood was made through the saphenous vein, which was exposed by a small skin incision. An exception to this site was made in the rats at and before 8 days of age; in them the donor blood was injected through the jugular vein. A specially calibrated 0.25-milliliter tuberculin syringe was used, so that a constant volume of donor blood was injected into each animal. At various times during the course of the blood-volume determinations the calibrated syringe was used to put the same volume of donor blood as was injected into each animal into a 10-ml volumetric flask. The blood was then diluted and an aliquot used for determining the amount of radioactivity injected into each animal. After injection, 6 minutes were allowed for the donor blood to mix, at the end of which time the abdomen was opened, and as much blood as would flow freely was drawn from the bena cava into a heparinized syringe. This usually amounted to about half the blood volume. In the rats at or younger than 15 days of age the blood was drawn from the aorta or the heart. A portion of this blood was used for an hematocrit determination and an aliquot was counted directly in a scintillation counter adapted for vial counting.[6] Hemoglobin determinations were done by the method of Turner.[110] The amount of radioactivity injected was divided by the amount of radioactivity per unit of recipient blood, to obtain the blood volume. The total blood volume multiplied by the hematocrit gave the total red cell volume,

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* The diet was obtained from Simonsen Laboratories, Gilroy, California. It consisted of 59.0% wheat, 11.7% skim milk, 11.2% casein, 11.2% rice bran, 3.3% vegetable oil, 1.3% $CaCO_3$, 0.7% NaCl, and vitamin and mineral mixtures to make up 100%.

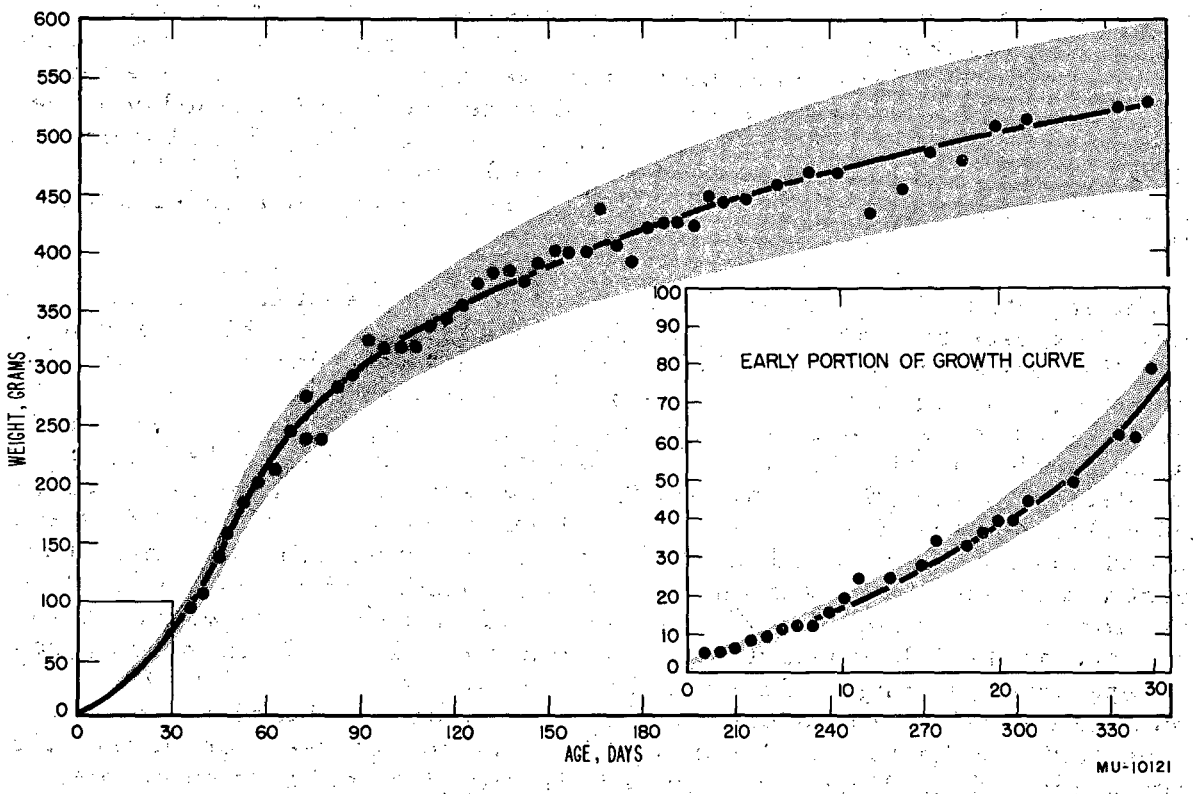

Fig. 1. Growth curve for male rats of the Long-Evans strain in the stock colony of the Donner Laboratory.

and the total plasma volume was taken to be the difference between the total blood volume and the total red cell volume. The total circulating hemoglobin was obtained by multiplying the total blood volume by the hemoglobin concentration. The blood, plasma, and red cell volumes were then corrected for the small volume of donor blood, plasma, and red cells injected. The blood, plasma, and red cell volumes are presented as ml per 100 g body weight; hemoglobin is presented similarly in g per 100 g body weight.

During the course of the study it became evident that certain criticisms might be brought against the Fe^{59}-labeled-cell technique as presented here for blood volumes. Among these are the possibility of agglutination reactions from injection of the donor blood, and the question whether or not the red cells in the spleen mix with the donor cells and so are included in the total red cell volume determination and also the question of the validity of the blood and plasma volume when a red cell diluent is used (rather than a plasma diluent).

To check the possibility of errors in the Fe^{59}-labeled-cell dilution technique due to agglutination reactions, the blood taken from 20 rats from the stock colony was centrifuged and the plasma was separated. A sample of the cells of each animal was cross-matched with a sample of the plasma of each of the other rats, giving rise to total of 380 cross-matchings. In no case was any evidence of agglutination observed; hence, this was ruled out as a possible source of error.

Reeve et al. showed in the dog that P^{32}-tagged cells were completely mixed with the red cells trapped in the spleen and that the ratio of cells to radioactivity remained the same even after adrenalin injection.[96] Such an animal as the dog might be expected, because of its relatively large spleen, to offer a greater problem than the rat with respect to complete mixing of tagged cells with those red cells sequestered in the spleen.

An attempt was made to study the problem of mixing of labeled red cells in the rat. Three male rats, weighing approximately 400 grams, were anesthetized with ether, and blood samples were collected continuously via jugular cannulation. After the beginning of the collection of blood samples the animal was injected via the saphenous vein with a known volume of Fe^{59}-tagged red cells. The collection of blood samples was carried on for 60 to 75 minutes after the injection of the tagged cells. A total of 15 to 20 blood samples of approximately 0.75 ml each was taken throughout this period. An aliquot of each blood sample was counted for radioactivity and a hemoglobin determination was made. As can be seen in Fig. 2, the concentration of hemoglobin decreased and the calculated total blood volume increased continuously throughout the period of bleeding. As a result of this relatively large loss of red cells from the circulation, one would expect, if the spleen had red cells trapped, that these cells would be released into the circulation. Furthermore, if these cells had not mixed with the Fe^{59}-tagged red cells, the ratio of radioactivity to hemoglobin would be changed, and the calculated total circulating hemoglobin would show an increase.

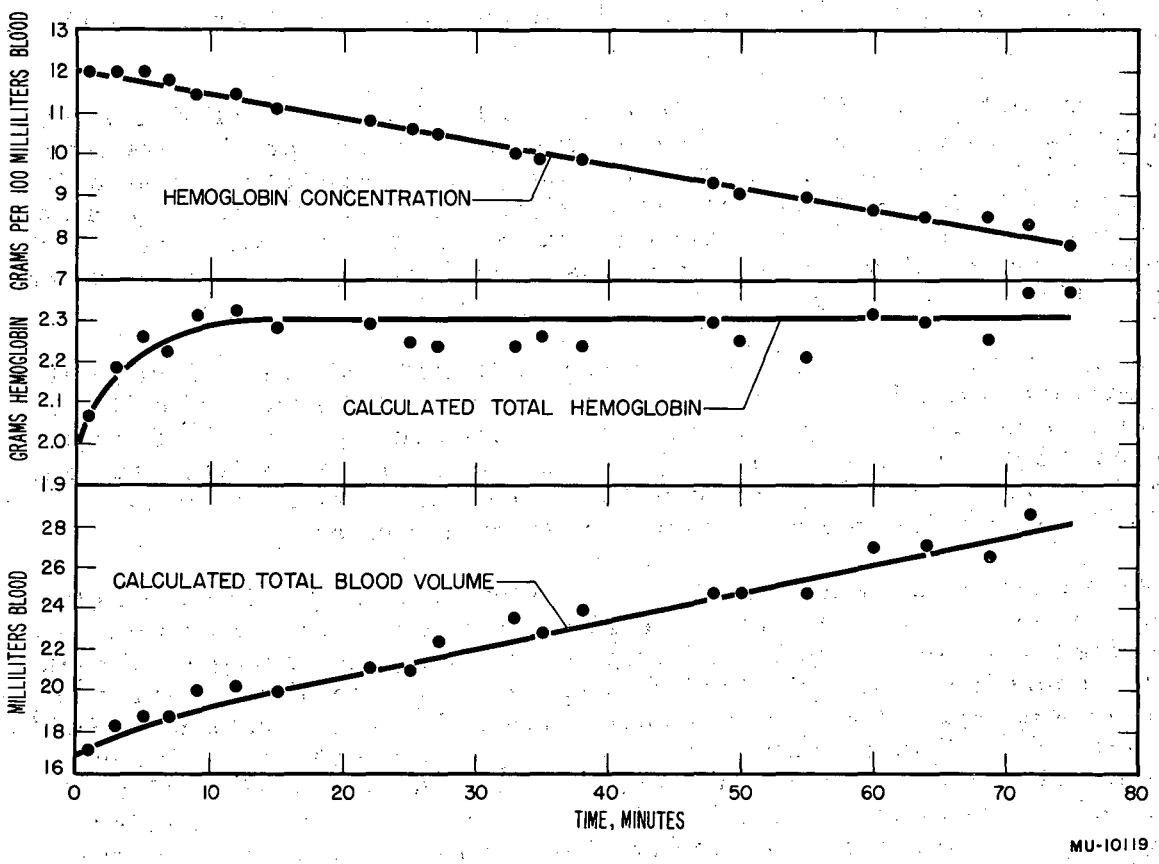

Fig. 2. Hemoglobin concentration, total hemoglobin, and blood-volume changes in the rat throughout continuous bleeding via jugular cannulation. Each point represents approximately 0.75 ml of blood.

This is not the case, as can be seen from Fig. 2. The calculated total circulating hemoglobin remains relatively constant in spite of the large loss of red cells from the circulation.

The errors encountered in making blood-volume determinations by the use of either a plasma diluent or a red cell diluent alone have been pointed out by Reeve et al.[96] The author agrees that the plasma volume should be measured by the use of a plasma diluent and that the red cell volume should be measured by use of a red cell diluent, and that the blood volume should be considered only as the sum of these two determinations. However, in the blood-volume determinations made in this study, a large volume of blood was drawn--in most cases, more than half the total blood volume. It was thus felt that the hematocrit determination, made on such a large portion of the animal's total blood volume, was probably very close to the average hematocrit in the body as a whole and that the blood and plasma volume determination calculated therefrom would have a dependable degree of accuracy.

The rats used for the blood-volume study as a function of age were males of the Long-Evans strain picked at random from the stock colony. Groups of 10 to 18 rats varying in age from 1 to 340 days were used. The numbers and ages of rats used in each group are given in Table I.

Results and Discussion

Table I is a complete tabulation of the average data for each group of rats used in the blood-volume study as a function of age. Hemoglobin concentrations and hematocrit values have been plotted in Fig. 3. Both these determinations remain relatively constant throughout the greater portion of the age period studied (70 to 340 days of age); the hematocrit remains at about 45 ml of red-blood cells per 100 ml of blood and the hemoglobin concentration at approximately 13 g of hemoglobin per 100 ml of blood. However, as has been noted by other authors an anemia exists prior to this period.[14, 29, 66, 68] From the hematocrit and hemoglobin values, it would appear that this anemia reaches a maximum at 15 days of age in the Long-Evans male rat. At this time the hematocrit falls to approximately 20 ml of red blood cells per 100 ml of blood, and the hemoglobin concentration falls to nearly 5 g of hemoglobin per 100 ml of blood.

The blood, plasma, and red cell volumes per unit body weight are given in Fig. 4. The unit blood volume* shows a continuous fall with increasing age from a value of approximately 7.2 ml per 100 g body weight in the newborn rat to a plateau level at about 4.4 ml per 100 g after 200 days of age. The unit plasma volume shows an increase from a value of approximately 4.6 ml per 100 g body weight at birth to a value of 5.3 ml between 8 and 15 days of age. In order to test the significance of this increase, the unit plasma-volume values from the data at 1 and 4 days were grouped, and statistically compared with the grouped values obtained at 8 and 15 days of age. Fisher's "T" test[35] was employed, and in this case gave a "p" value of less than 0.001, thus indicating

* The term "unit volume" is used to denote volume per unit body weight.

Table I

Hematological data from normal male rats as a function of age

No. of Rats	Age (days)	Wt. (g)	Hct. (%)	Hb. g / 100 ml	TBV (ml)	TPV (ml)	TRCV (ml)	THb. (g)	BV ± SD ml / 100 g body weight	PV ± SD ml / 100 g body weight	RCV ± SD ml / 100 g body weight	Hb. ± SD g / 100 g body weight
10	1	6.5	37.7	11.1	0.47	0.30	0.17	0.051	7.22 .38	4.57 .39	2.65 .32	0.783 .111
12	4	10.0	33.2	8.2	0.68	0.46	0.22	0.054	6.82 .61	4.64 .59	2.18 .31	0.536 .067
14	8	15.9	25.1	6.6	1.10	0.84	0.26	0.068	6.92 .41	5.26 .61	1.66 .31	0.435 .093
14	15	29.2	20.2	5.2	1.97	1.59	0.38	0.097	6.73 .50	5.42 .47	1.31 .21	0.334 .057
11	21	46	25.2	6.5	2.77	2.09	0.68	0.174	6.02 .49	4.55 .54	1.47 .10	0.377 .020
12	25	55	23.8	5.7	3.51	2.70	0.81	0.194	6.36 .34	4.89 .34	1.46 .14	0.349 .042
13	34	86	28.5	7.2	5.19	3.70	1.49	0.377	6.05 .31	4.33 .39	1.72 .21	0.432 .066
10	44	135	32.9	8.9	7.96	5.33	2.63	0.712	5.88 .45	3.94 .36	1.94 .32	0.526 .071
10	64	249	42.3	11.3	12.63	7.28	5.35	1.42	5.08 .17	2.93 .27	2.15 .15	0.573 .044
12	72	205	43.0	12.6	10.59	6.03	4.56	1.34	5.19 .26	2.95 .18	2.23 .21	0.653 .066
10	84	264	45.8	12.8	12.87	7.01	5.86	1.65	4.88 .34	2.65 .30	2.23 .16	0.628 .061
10	104	255	43.0	12.7	12.72	7.23	5.49	1.62	5.00 .32	2.85 .17	2.15 .21	0.635 .061
11	114	352	45.5	13.2	16.78	9.15	7.63	2.22	4.77 .23	2.60 .16	2.17 .11	0.631 .032
10	138	397	46.3	14.0	18.54	9.95	8.59	2.61	4.67 .28	2.51 .13	2.16 .17	0.657 .055
18	161	409	46.4	13.6	18.64	9.99	8.65	2.54	4.55 .22	2.44 .21	2.11 .11	0.621 .032
10	164	366	48.8	13.3	16.18	8.28	7.90	2.14	4.42 .30	2.27 .33	2.15 .21	0.587 .033
10	192	398	44.7	13.3	17.87	9.85	8.03	2.38	4.48 .14	2.48 .12	2.00 .14	0.595 .037
10	222	427	45.0	12.4	18.69	10.27	8.42	2.33	4.38 .24	2.41 .17	1.97 .14	0.544 .035
10	229	465	44.1	12.4	21.19	11.85	9.34	2.63	4.55 .18	2.55 .13	2.00 .07	0.565 .018
10	245	483	45.1	13.8	20.64	11.34	9.31	2.85	4.27 .19	2.34 .15	1.93 .06	0.591 .020
10	264	430	44.6	12.9	17.96	9.94	8.02	2.32	4.19 .26	2.32 .13	1.87 .16	0.542 .038
12	309	514	45.2	12.9	23.74	13.02	10.71	3.02	4.62 .18	2.53 .15	2.09 .09	0.596 .025
10	340	573	45.3	12.5	25.68	14.06	11.62	3.20	4.51 .42	2.47 .34	2.03 .12	0.561 .048

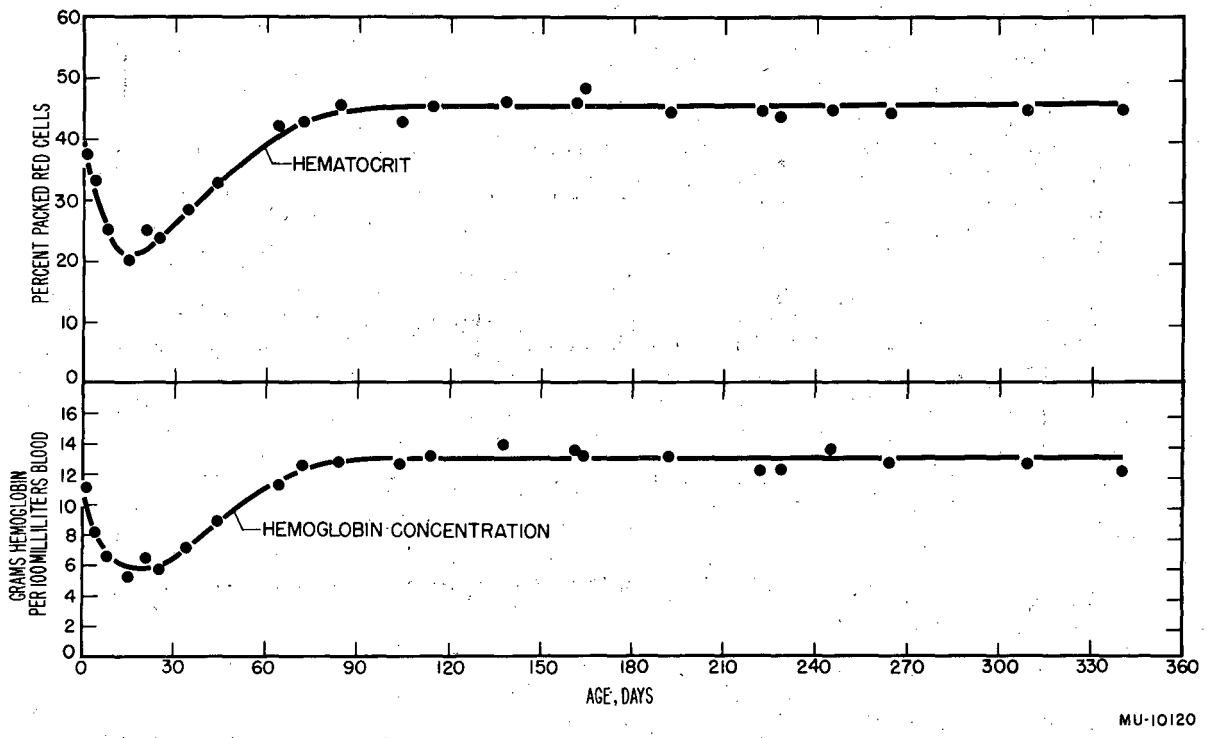

Fig. 3. Hematocrit and hemoglobin concentration as a function of age in normal male rats.

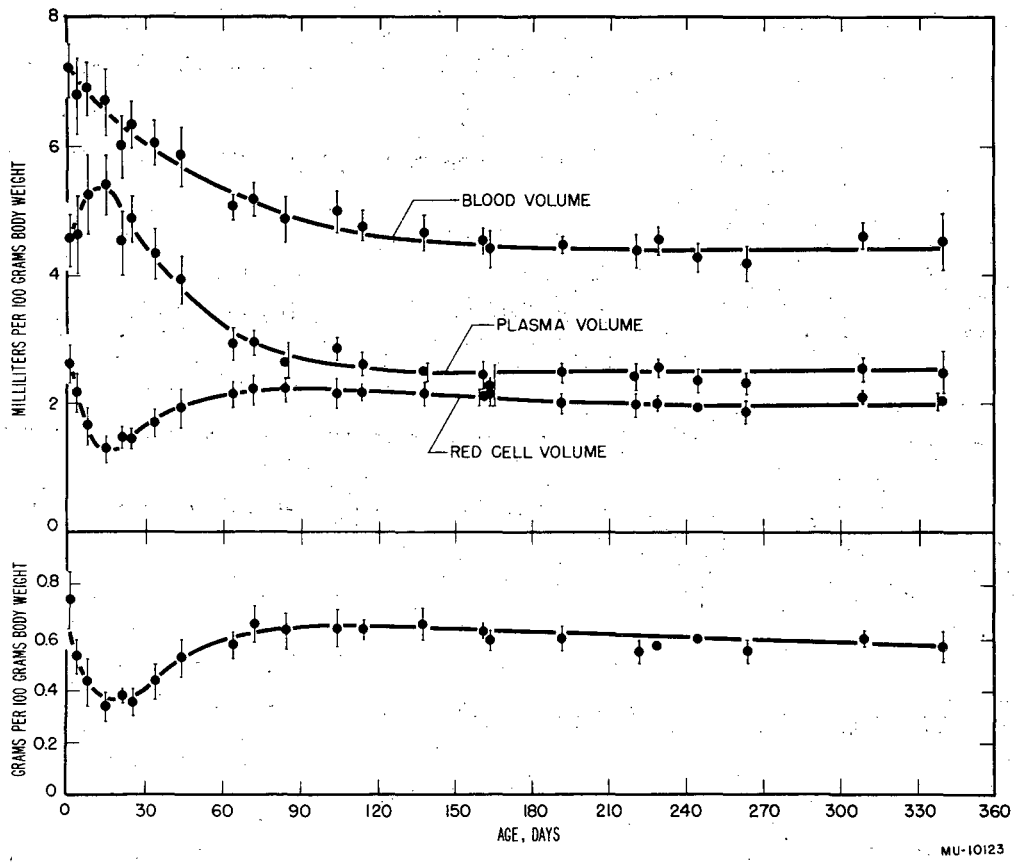

Fig. 4. Blood volume, plasma volume, red cell volume, and hemoglobin per 100 g body weight as a function of age in normal male rats.

a significant increase in the unit plasma volume from birth to 15 days of age. In part at least, the fall in hematocrit and hemoglobin concentration may be due to hemodilution. Beyond 15 days of age, the unit plasma volume falls continuously to a plateau level after 200 days of age of approximately 2.4 ml per 100 g body weight.

The unit red cell volume remains relatively constant from 60 to 150 days at approximately 2.2 ml red blood cells per 100 g body weight. This period is followed by a gradual decrease in the unit red cell volume to approximately 2.0 ml per 100 g body weight. In order to test the significance of this drop, all the unit red cell volumes measured on animals between 60 and 160 days of age were grouped and statistically compared with the group of values obtained in animals over 200 days of age. This gave a "p" value of less than 0.001 and so is considered as a significant drop.

Bone growth of Long-Evans male rats in our colony measured by x-ray films indicates a plateau in bone growth, which is reached at approximately 150 days of age. Nevertheless, after this age, as can be seen from the growth curve in Fig. 1, the animals continue to gain in weight. The gradual decrease in unit red cell volume after 160 days of age possibly is due to an increase mainly in body fat without a relative increase in red cell volume.

Prior to 60 days of age in the Long-Evans male rat there is a definite decrease in the unit red cell volume, with the lowest values reached at approximately 15 to 20 days of age. One day after birth the unit red cell volume is about 2.6 ml of red blood cells per 100 g body weight. This value falls rapidly to a level of approximately 1.3 ml at 15 to 20 days of age and then returns slowly to 2.2 ml at 60 days of age. In order to test the significance of this decrease in unit red cell volume, the values obtained from 1 to 4 days of age were grouped and statistically compared with those values obtained between 15 and 21 days of age. This gave a "p" value of less than 0.001 and therefore can be considered significant. The group of values on rats between 15 and 21 days of age is also significantly different from the groups of values between 60 and 160 days of age with a "p" value of less than 0.001.

The plot of unit hemoglobin (also given in Fig. 4) is very similar to the curve for unit red cell volume. The unit hemoglobin drops from a value of 0.78 g hemoglobin per 100 g body weight at 1 day of age to approximately 0.35 g per 100 body weight between 15 and 25 days of age, after which it rises to a level of approximately 0.64 g per 100 g body weight. After 200 days of age the unit hemoglobin falls to a level of approximately 0.57 g per 100 g body weight.

The anemic period thus existing in the rat following birth is truly an anemia in every sense of the word. Both the hemoglobin concentration and hemocrit values are greatly reduced in this period, although perhaps exaggerated to a slight extent by a relatively increased plasma volume at the same time. Most convincing argument for this anemia is that the amount of hemoglobin and the red cell volume relative to the body mass are significantly reduced in this period.

It has been pointed out that the anemia of the newborn is possibly the result of a lack of erythropoietic stimulation.[22,69] However, it appears to the author, on considering the very rapid relative growth rate of the body mass as a whole that follows birth, that the anemia which exists at this time is perhaps simply a reflection of an inability of the marrow output of red blood cells to keep up with this very rapid growth and therefore for a period lags behind the body growth as a whole. If such were the case, then the anemia could not be the result of a lack of erythropoietic stimulation, since the bone marrow would be producing red cells at a maximum rate and even then would be unable to keep pace with body growth. Also, if maximum red cell production is taking place at this time, then it should be impossible to stimulate the bone marrow to greater red cell production. This question will be given further attention later in this study.

A curve showing the growth of the total red cell volume as a function of age is given in Fig. 5-A. This was constructed by using the growth curve given in Fig. 1 and unit red cell volume (ml red blood cells per 100 g body weight) given in Table I. It can be seen from the curve that never is there seen a decrease in the total red cell volume, even during the anemic period. On the contrary, the curve shows a continual increase in red cell volume throughout the age range covered. It is pointed out here that the earliest observations made were one day after birth, and thus nothing can be said as to the changes of the red cell volume immediately following birth to day one. The continual increase in red cell volume is perhaps better depicted in Fig. 5-B, which is a plot, made from the curve of the total red cell volume, of the daily gain in red cell volume as a function of age. This curve indicates the fluctuations in the rate at which red cells are gained by the circulation. The gain in red cells per day increases rapidly until approximately 40 days of age, at which time the total red cell volume gains red cells at the rate of approximately 0.13 ml per day. After 40 days of age the rate of gain in red cells by the circulation decreases rapidly until approximately 90 days of age, and then decreases more slowly. Interestingly, throughout the anemic period the rat continues to gain red cells, and even at the peak of the anemia; that is, between 15 and 20 days of age, the animal gains red cells at the rate of approximately 0.03 ml of red cells per day.

If the data are carried one step further, as shown in Fig. 5-C, where the daily gain in red blood cells by the circulation is divided by the weight of the animal, a curve is obtained which gives the gain in red blood cells per day, per gram of animal, as a function of age. This curve perhaps best indicates the daily amount of energy put into the growth of the red cell volume relative to the growth of the animal as a whole. From birth to 40 days of age the gain in red blood cells per gram of rat per day varies within 10% of 0.001 ml. This value falls rapidly, so that at 60 days of age it is approximately one-half and at 100 days is almost one-tenth that which exists at and before 40 days of age. It is realized that this curve does not include the production of red cells by the bone marrow to replace those which are destroyed, but it can be interpreted as the daily production of red cells per gram of animal over that necessary to maintain the red cell volume in the face of the normal red cell destruction. Thus the daily production of red

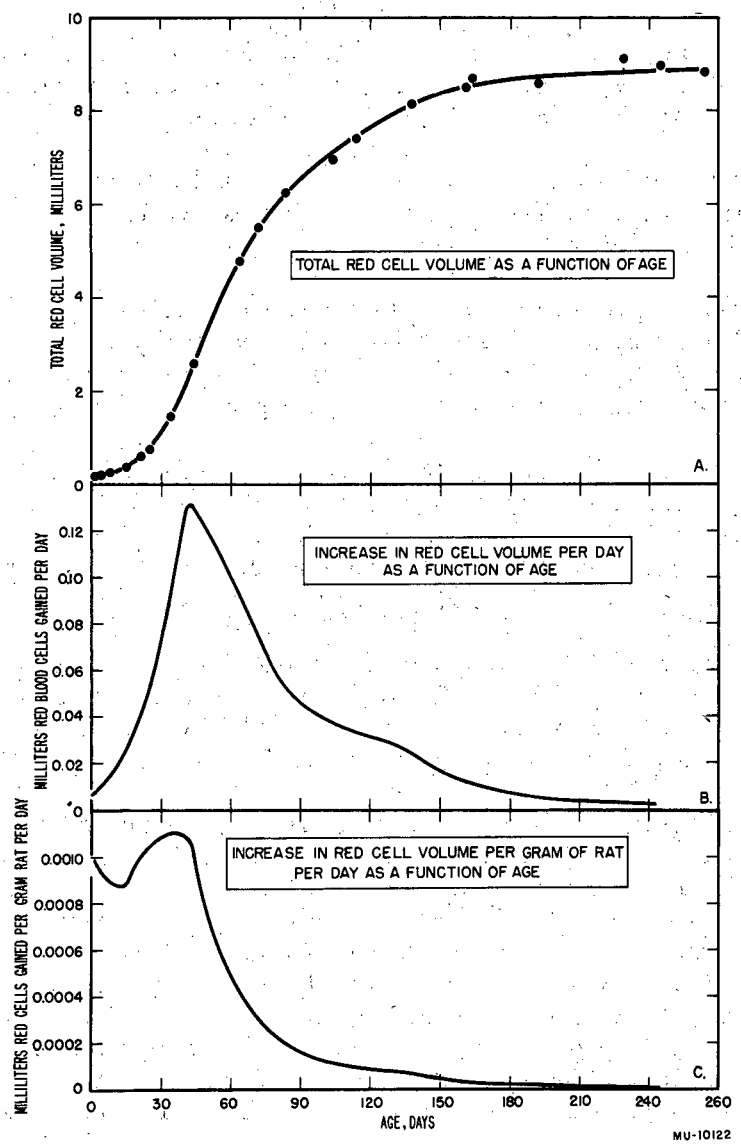

Fig. 5. Variations in red cell volume with age.

cells per gram of animal in excess of the maintainance production is highest in the rat from birth to 40 days of age, covering the period of anemia in the rat. The lack of erythropoietic stimulation certainly cannot then be implicated as a cause of the anemia.

Summary

By use of the Fe^{59}-labeled-cell dilution technique, the blood, plasma, and red cell volume and total circulating hemoglobin have been determined on 259 male rats varying in age from 1 to 340 days.

The unit blood volume in the male rat falls continuously with age from a value of approximately 7.2 ml per 100 g body weight at 1 day of age to a level of 4.4 ml per 100 g body weight after 200 days.

There is a significant increase in the unit plasma volume in the male rat from a value of approximately 4.6 ml per 100 g body weight at 1 day of age to a value of 5.3 at 15 days. The unit plasma volume then falls continuously to a level of approximately 2.4 ml per 100 g body weight after 200 days.

In the male rat the unit red cell volume falls significantly from 2.6 ml per 100 g body weight at 1 day of age to a value of 1.3 at 15 to 20 days. The unit red cell volume then increases slowly to a level of 2.2 ml per 100 g body weight between 60 and 160 days of age and then decreases to a level of 2.0 ml per 100 g body weight after 200 days.

The unit hemoglobin falls from 0.78 g per 100 g body weight at 1 day of age to 0.35 g between 15 and 25 days and then increases to a value of 0.64 g per 100 g body weight at approximately 70 days. After 200 days of age the unit hemoglobin drops to a value of 0.57 g per 100 g body weight.

An anemic period exists in the male rat which is maximal between 15 and 20 days of age. This anemia is observed in terms of a decrease in the volume of red blood cells per gram of rat as well as in hematocrit and hemoglobin concentration values. Throughout this anemic period, however, there is a continual increase in the total red cell volume, and, in fact, the daily gain of red blood cells per gram of rat is greater during this period than at any time after. In addition to red cells produced to replace those which are destroyed, approximately 0.001 ml of red cells are produced per g of animal per day throughout the anemic period. Thus it is concluded that the anemia observed is not due to a lack of erythropoietic stimulation.

STUDIES OF THE ERYTHROPOIETIC RESPONSE TO HYPOXIA AS A FUNCTION OF AGE

Since Bert found a polycythemia in animals exposed to low barometric pressures,[12] this has been regarded as an important stimulus to erythropoiesis. Indeed, Grant and Root, in a review of the subject, consider anoxia as the fundamental or primary stimulus for erythropoiesis.[45] Many other authors have noted the increase in hematocrit, hemoglobin concentration, and red cell count in animals as well as in man in response to anoxia.[10, 108, 106] Schneider, in a review of the early literature, concludes that there is a real increase in total circulating red cells at altitude, and that the blood changes observed are not merely due to concentration of the blood.[101] Dallwig et al. observed that the blood changes that occur at altitude also appear in animals in an environment of reduced partial pressure of oxygen at normal barometric pressure.[26] These authors conclude that the erythropoietic response observed at high altitude is largely, if not entirely due, to the decreased partial pressure of oxygen. Campbell also observed an increased red cell count and hemoglobin concentration in animals placed in an environment of reduced partial pressure of oxygen alone.[17] He also noted that an increased partial pressure of oxygen above the normal value results in a reduced red cell count and hemoglobin concentration in animals.

An increased reticulocyte percentage has been observed by many authors in response to altitude.[42, 63, 103, 116] Hyperplasia of the erythroid elements in the bone marrow[116] and an increase in the number of mitotic figures in the bone marrow[44] have also been observed. That the spleen plays an erythropoietic role under the stress of hypoxia has been observed by Rambach et al.[93] Erythropoiesis in the spleen also occurs along with the polycythemia that follows cobalt injection.[28] Huff et al. observed in man that upon movement to a high altitude, the rate of iron turnover increased by a factor of two.[63]

An increased blood volume has been observed at high altitude.[7] However, the increase in blood volume is small in comparison with the increased red cell volume observed.[38, 97, 112] In part the increased red cell volume observed is compensated for by a decreased plasma volume.[38, 60, 63, 97] Fryers and Berlin conclude that the average red cell life in altitude acclimatized rats is essentially normal.[39]

Several authors have questioned the physiological value of increased red cell volumes at altitude.[18, 59] However, others have observed increased work performance and altitude tolerance in altitude-acclimatized animals[10, 53] and in animals made polycythemic with cobalt,[28] as well as by artifical polycythemia produced by transfusion.[10, 92]

That an erythropoietic response due to an intermittent hypoxia can be observed has been substantiated by many authors.[3, 4, 107, 109] Increased red cell volumes,[112] erythroid hyperplasia of the bone marrow,[32] and increased numbers of mitotic figures[44] have been observed in response to intermittent hypoxia.

The study reported herein is directed mainly to the investigation of the erythropoietic response to an hypoxic stimulus in rats as a function of age. Exposure to an intermittent hypoxia, produced by reducing the tension of oxygen by reducing its concentration and maintaining normal barometric pressure, is used.

Methods

A lucite chamber approximately 3 ft long, 2 ft wide, and 2 ft high, with a gastight opening at one end, was used for the hypoxic environment. As depicted in Fig. 6, the chamber was furnished with two intake and two exhaust openings. The main exhaust was left open to the outside of the chamber. The other exhaust was passed through a fiberglass wool filter and then through a Beckman Oxygen Analyzer (Model E-2) from which a continuous measure of the oxygen concentration could be obtained. Attached to one of the intakes by a Y tube were a cylinder of compressed nitrogen and a cylinder of compressed air, each equipped with gas-flow regulators. The desired oxygen concentration was maintained by adjustment of the flow of nitrogen and air from these cylinders. To the other intake was attached a cylinder of compressed nitrogen, used only at the beginning of each hypoxic period to lower the concentration of oxygen within the chamber to the desired level.

The chamber was equipped with a blower in order to keep the gas within the chamber mixed. Also, to absorb the carbon dioxide, a petri dish containing "Ascarite" was placed within the chamber.

The animals to be studied in the hypoxic environment were placed in the chamber and the door was sealed. Nitrogen from the single cylinder was allowed to enter the chamber at a relatively rapid rate until the oxygen concentration required, as read from the oxygen analyzer, was reached. The valve on this nitrogen cylinder was then closed and the valves on the other nitrogen cylinder and the compressed air cylinder were opened. The flow of gas from each of these cylinders was adjusted by the attached flow regulator so that the required oxygen concentration was maintained. It was found after the initial adjustment on these valves that very little further adjustment was required throughout the 6-hour period. In fact, if the same animals were placed in the chamber the following day practically no adjustment of these valves was necessary after the initial lowering of the oxygen tension within the chamber. This procedure took a total of 10 to 15 minutes. The total gas flow required to maintain the hypoxic environment was between 5 and 8 liters per minute.

The control rats were kept at atmospheric oxygen concentration.

Groups of male Long-Evans rats from 5 to 250 days of age were divided into experimental and control groups, each having the same average weight. Animals below the weaning age of 21 days were also divided so that each litter contributed the same number of animals to the control and

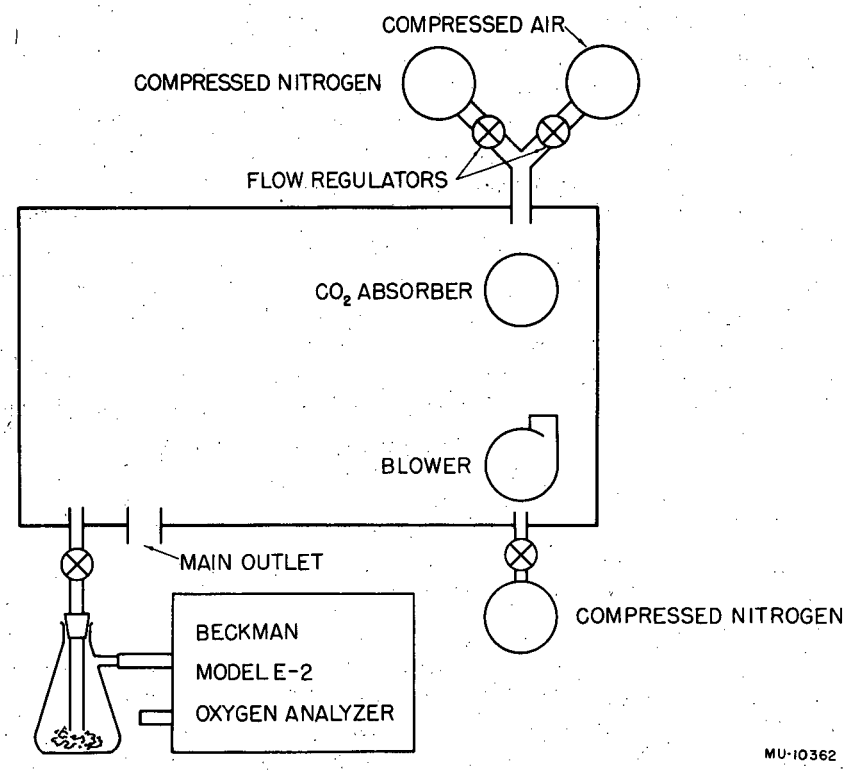

Fig. 6. Schematic diagram of the apparatus used to obtain an environment of low oxygen tension.

experimental groups. The experimental groups were exposed to a hypoxic environment of 9.0% oxygen 6 hours daily for 14 days. Animals below weaning age were then placed with their mothers for the remaining 18 hours. The control animals below the weaning age were also removed from their mothers for the daily 6-hour period.

Red cell volume determinations were done by means of the Fe^{59}-labeled red cell dilution technique as given earlier.

Results and Discussion

It has become customary to give the blood, plasma, or red cell volume in terms of ml per unit of body weight. Such a measure as the simple relationship of the red cell volume to the body weight is useful, since it does give one an idea of the relative amount of red cells per unit of body weight for an animal in a given situation. Here, however, the labeled red cell volume technique was used to give a measure of the production of red cells in response to an hypoxic stimulus. It was observed from previous studies that many times, as a result of an hypoxic environment, animals would lose weight or young animals would fail to gain weight as their controls did. In this situation it became obvious that the possibility of a serious error was introduced when the unit red cell volume of a control was compared with the unit red cell volume of an experimental animal. If an experimental animal loses weight, the unit red cell volume shows an increase when compared with its control. It can be seen how such stimulation can be exaggerated when one is attempting to determine the degree of erythropoietic stimulation as the result of some experimental procedure. Indeed, if the loss of weight were great enough, an erythropoietic response might be assumed to exist where there is really no net increase in the total number of circulating red blood cells.

In a crude attempt to show such a situation, two groups of male Long-Evans rats approximately 200 days of age were fasted for a period of time and then their red cell volumes were measured. Both groups were divided equally so that the average beginning weights were as close as possible in the control and fasted animals. One group was fasted for a 6-day period. The other group was fasted for a 12-day period with food being given every fourth day. The hematological results are given in Table II. It can be seen that in both of the fasted groups a considerable amount of weight was lost. Also, it can be seen that in spite of a significant increase in the red cell volume and hemoglobin per 100 g body weight, the total volume of red cells and amount of hemoglobin actually shows a net decrease. Although the red cell volume and hemoglobin per 100 g body weight shows a significant increase, such a precedure as fasting could by no means be interpreted as an erythropoietic stimulation.

Table II

Hematological changes in male rats following fasting

	No. of Rats	Wt. Begin (g)	Wt. End (g)	Hct. (%)	Hb. (g / 100 ml)	TBV (ml)	TRCV (ml)	THb. (g)	RCV (ml / 100 g body weight)	Change (%)	p	Hb. (g / 100 g body weight)	Change (%)	p
Control	10	424	427	45.0	12.4	18.69	8.42	2.33	1.97			0.544		
										13.7	<.01		14.3	<.01
Fasted (6 days continuously)	10	427	357	50.4	14.0	15.89	8.00	2.22	2.24			0.622		
Control	10	460	465	44.1	12.4	21.19	9.34	2.63	2.00			0.565		
										15.5	<.001		18.2	<.001
Fasted (12 days, fed every 4 th day)	10	463	359	47.0	13.6	17.56	8.25	2.39	2.31			0.668		

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As a result of this study, it was concluded that if the object of an experiment was to determine whether erythropoietic stimulation resulted in response to some stimulus, then the red cell volume per unit body weight could not be used, but a significant increase in the total red cell volume must be observed. The same reasoning can be applied to animals that do not lose weight, but fail to gain in weight as do their controls; for if they do not accumulate a net increase in total red cell volume over their controls, then it cannot be assumed that they were erythropoietically stimulated to any greater extent than were their controls. This is not meant to say that in all situations where the unit red cell volume increases, no erythropoietic stimulation exists; but only that if no net increase in the total red cell volume has accrued the red cell volume technique has been used to its limit and thus nothing can be assumed regarding erythropoietic stimulation.

Table III is a tabulation of the hematological changes resulting in Long-Evans rats after a daily 6-hour exposure to an environment of 9.0% oxygen. Groups of rats of 5, 10, 20, 30, 50, 70, 100, 150, and 250 days of age were exposed to this environment for a period of 14 days. At approximately 18 hours after the last exposure the Fe^{59}-labeled red cell dilution technique was employed to determine the blood changes in these animals as compared with their controls. A minimum of twenty animals were involved at each age studied; ten controls and ten animals exposed to the hypoxic environment. It will be seen from Table III that the average weight of the control and hypoxic groups are very close at the beginning of the experiment. At the end of the 14-day period, in all of the ages studied, the average weight of the hypoxic group was less than the average weight of the control group.

The hematocrit and hemoglobin concentration are increased as a result of the hypoxic environment in all the ages studied. In part this is perhaps a reflection of the reduced total plasma volume seen in the hypoxic animals.

In Table III the average total red cell volumes and total hemoglobins for the hypoxic animals and their controls for all the ages studied are given. Also, the percentage change and the "p" values for these data are calculated. The values for total red cell volume and total hemoglobin of the hypoxic animals are not significantly different from the control values for the four youngest ages studied; that is, the animals beginning their 14-day hypoxia at 5, 10, 20, and 30 days of age. Of the ages studied, the hypoxic rats beginning their 14-day hypoxia at 50 days of age are the youngest animals to show a significant increase in the total red cell volume and total hemoglobin over the control values. In all the groups of hypoxic rats older than this group, the total red cell volumes and total hemoglobins are significantly increased over those in their respective control groups.

Thus it appears that rats 50 days of age or older are able to respond well erythropoietically to an hypoxia of 9.0% oxygen for 6 hours daily for 14 days. However, the rats of younger ages studied did not show a significant increase in total red cell volume and total hemoglobin when exposed to this stimulus. This is interpreted as indicating an inability of these young rats to respond erythropoietically to the hypoxic stimulus used. Such a result

Table III

Hematological changes in male rats of varying ages exposed to 9.0 percent oxygen, 6 hours daily for 14 days

	Age (days)	No. of rats	Wt. begin (g)	Wt. end (g)	Hct. (%)	Hb. (g /100 ml)	TBV (ml)	TPV (ml)	TRCV (ml)	Change (%)	p	THb. (g)	Change (%)	p
Control	5-19	10	11.6	35.8	23.6	6.2	2.27	1.74	0.53			0.139		
Hypoxic	"	10	11.3	31.2	27.2	6.9	2.03	1.49	0.54	1.9	.8	0.138	0.7	.9
Control	10-24	10	19.7	52.9	24.8	5.8	3.54	2.66	0.88			0.206		
Hypoxic	"	10	19.9	47.8	25.5	5.9	3.28	2.45	0.83	-5.2	.5	0.194	-5.8	.8
Control	20-34	13	41.2	86	28.5	7.2	5.19	3.70	1.49			0.377		
Hypoxic	"	13	41.2	75	34.6	8.1	4.77	3.11	1.66	11.4	.3	0.388	2.9	.8
Control	30-44	10	79	135	32.9	8.9	7.96	5.33	2.63			0.712		
Hypoxic	"	10	78	109	40.2	10.1	7.24	4.31	2.92	11.0	.2	0.735	3.2	.8
Control	50-64	10	188	249	42.3	11.3	12.63	7.28	5.35			1.42		
Hypoxic	"	10	188	223	53.6	13.2	12.59	5.78	6.81	27.3	<.01	1.67	17.6	<.05
Control	70-84	10	237	264	45.8	12.8	12.87	7.01	5.86			1.65		
Hypoxic	"	10	237	249	52.7	14.7	14.06	6.64	7.42	26.6	<.001	2.08	26.1	<.001
Control	100-114	11	333	352	45.5	13.2	16.78	9.15	7.63			2.22		
Hypoxic	"	11	335	333	55.5	15.6	17.90	7.92	9.99	31.0	<.001	2.81	26.6	<.001
Control	150-164	10	362	366	48.8	13.3	16.18	8.28	7.90			2.14		
Hypoxic	"	10	363	355	58.1	15.5	18.01	7.56	10.45	32.3	<.001	2.79	30.4	<.001
Control	250-264	10	439	430	44.6	12.9	17.96	9.94	8.02			2.32		
Hypoxic	"	10	438	420	62.1	15.6	20.69	8.03	12.66	57.9	<.001	3.24	39.7	<.001

was not unexpected, since these young animals are in such a high state of erythropoiesis in attempting to meet the great demands of body growth at this time. These data are interpreted as a further indication of the high erythropoietic state existing in young animals and that the red cell production is at a maximal or near maximal rate at this time and cannot be further stimulated.

Grant reported the occurrence of an increased total body hemoglobin in young rats that were nursed by intermittently hypoxic mothers.[43] It was thought pertinent that such an experiment should be repeated in the light of the foregoing evidence of an inability of the marrow of young rats to respond to direct hypoxia by increased red cell production. Six lactating Long-Evans rats were used; three were exposed to intermittent hypoxia and three were used as controls at normal oxygen tension. Each of these mothers had a litter of four males, 6 days of age at the beginning of the study. At this time two of the young rats from each experimental mother were exchanged with two young rats of the control mothers. These were picked so that the average weight of the rats to be nursed from the hypoxic mothers was the same as the average weight of the rats nursed by the control mothers. The hypoxic stimulus used was again an intermittent daily 6-hour exposure to 9.0% oxygen for 14 days. The control mothers were removed from their young for the same 6-hour period daily. At the end of the 14-day period, the labeled red cell volume was determined on the young rats as well as on their mothers. A tabulation of the average data of control and hypoxic mothers is given in Table IV. As is expected, it shows an increase in the total red cell volume and total hemoglobin as well as in the hematocrit and hemoglobin concentration of the hypoxic mothers. Table V is a tabulation of the hematological results in the young rats. Essentially no increase is seen in any of the hematological values. Thus the erythropoietic stimulation of young rats nursing from hypoxic mothers could not be repeated under the conditions given here; that is, with Long-Evans rats and with reduced oxygen concentration rather than reduced barometric pressure in order to obtain an environment of reduced oxygen tension.

In an attempt to compare the production of red blood cells in response to hypoxia with that seen in young rats, a study was done of the erythropoietic response to hypoxia as a function of time at the hypoxic environment. Male rats of the Long-Evans strain between 130 and 150 days of age were used for this study. As can be seen in Fig. 5, this is at a time when the gain in red blood cells by the circulation is very low. For this study a greater degree of hypoxia was used; namely, 7.0% oxygen for 6 hours daily until the time of sacrifice. A mortality rate of approximately 30% was observed in this study as a result of this degree of hypoxia.

Labeled-red-cell volumes were done on groups of rats from 1 to 20 days after the beginning of the daily 6-hour exposures to hypoxia; that is, the volumes were done 18 hours after the last 6-hour exposure. Five to eight experimental rats were used per point and 21 rats were done as controls.

Table IV

Hematological changes in lactating rats exposed to 9.0 percent oxygen, 6 hours daily for 14 days

	No. of rats	Wt. begin (g)	Wt. end (g)	Hct. (%)	Hb. (g / 100 ml)	TBV (ml)	TRCV (ml)	THb. (g)	RCV (ml / 100 g body weight)	Hb. (g / 100 g body weight)
Control	3	290	294	43.6	12.0	16.93	7.37	2.03	2.51	0.690
Hypoxic	3	280	256	55.3	15.0	16.55	9.20	2.48	3.59	0.969

Table V

Hematological data in young male rats nursed by mothers exposed to 9.0 percent oxygen, 6 hours daily for 14 days

	Age (days)	No. of rats	Wt. begin (g)	Wt. end (g)	Hct. (%)	Hb. (g / 100 ml)	TBV (ml)	TRCV (ml)	THb. (g)	RCV (ml / 100 g body weight)	Hb. (g / 100 g body weight)
Nursed by control female	6-20	11	12.7	42.4	19.0	5.1	2.79	0.52	0.137	1.22	0.324
Nursed by hypoxic female	6-20	11	12.4	38.5	20.7	5.2	2.39	0.47	0.118	1.24	0.308

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These data, including the various times of exposure and the number of rats at each time, are given along with the hematological results in Table VI.

From the value for red cell volume per 100 g body weight obtained in the controls, the total red cell volume was calculated for the experimental rats, using the beginning weight. This gave a calculated value for the total red cell volume of each animal before exposure to the hypoxic environment. This calculated total red cell volume was then compared with the measured total red cell volume determined after exposure to the hypoxic environment. The percent change was then calculated, and this was plotted in Fig. 7. Also plotted in Fig. 7 is the slope of the line indicating the daily percentage gain in red cell volume of normal rats at the peak of the anemic period. The normal rat at 15 days of age shows a daily increase in red cell volume of approximately 6.5% per day. When the experimental curve in Fig. 7 is compared with this slope, it is seen that at no time is the slope of the curve for the daily gain in red cells due to hypoxia any greater than that seen for the slope representing the daily gain in red blood cells in the young rats during their anemic period. This would indicate that the relative daily production of red blood cells in response to a severe hypoxic stimulus, as given here, is at no time greater than the relative daily gain in red blood cells which exists in young rats up to 40 days of age. It is thus suggested that the high rate of red blood cell production existing in young rats is maximal, or near maximal, and that the rat is unable to exceed this productive rate even when a strong hypoxic stimulus is applied.

It is realized that the bone marrow is also producing red blood cells to maintain the red cell volume not withstanding red cell destruction. The production of red blood cells in response to an hypoxic stimulus and the gain in red cell volume in young growing rats compared above is in addition to that basal production which replaces the red blood cells lost from the circulation by destruction.

Summary

The labeled red cell volume technique was used to determine the erythropoietic response to an hypoxic stimulus in groups of male Long-Evans rats of various ages from 5 to 250 days of age. An environment of 9.0% oxygen 6 hours daily for 14 days was used as the hypoxic stimulus. Whereas rats of 50 days of age or older responded well erythropoietically to this hypoxic stimulus, rats of 5 to 30 days showed no net increase in total red cell volume or total hemoglobin when exposed to this stimulus. Also, no net increase in total red cell volume was seen in young nursing rats when the mothers were exposed to an intermittent hypoxic stimulus. The daily increase in red cell volume in response to an intermittent hypoxia of 7.0% oxygen for 6 hours daily was determined in adult rats. When this was compared with the relative daily gain in red cell volume in young rats, it was found that the relative daily gain in the hypoxic adult rats was always less than that in the young rats.

Table VI

Hematological changes in adult male rats exposed to 7.0 percent oxygen, 6 hours daily for from 1 to 20 days

	No. of rats	Wt. begin (g)	Wt. end (g)	Hct. (%)	TBV (ml)	TRCV determined (ml)	TRCV * calculated (ml)	Change (%)	RCV (ml / 100 g body weight)
Control rats	21		356	46.3	17.18	7.94			2.23
Hypoxic rats									
1 day	5	341	342	51.0	16.34	8.12	7.61	6.8	
2 days	5	351	342	50.8	16.20	8.23	7.83	5.1	
3 days	8	398	387	48.2	19.49	9.40	8.88	5.9	
4 days	5	326	310	50.5	16.01	8.10	7.27	11.4	
5 days	8	333	315	49.8	16.61	8.25	7.43	11.0	
6 days	5	429	397	54.0	20.63	11.13	9.57	16.3	
7 days	8	380	352	51.8	18.74	9.74	8.47	15.0	
8 days	7	370	341	54.4	17.63	9.57	8.25	16.0	
9 days	6	350	334	51.8	18.16	9.38	7.81	20.1	
11 days	6	405	381	54.9	20.38	11.26	9.03	24.7	
15 days	5	410	389	56.0	21.39	11.99	9.14	31.2	
20 days	6	405	348	57.9	20.97	12.15	9.03	34.6	

* Calculated TRCV is the expected TRCV at the beginning of the experiment, based on the control value for RCV per 100 g body weight.

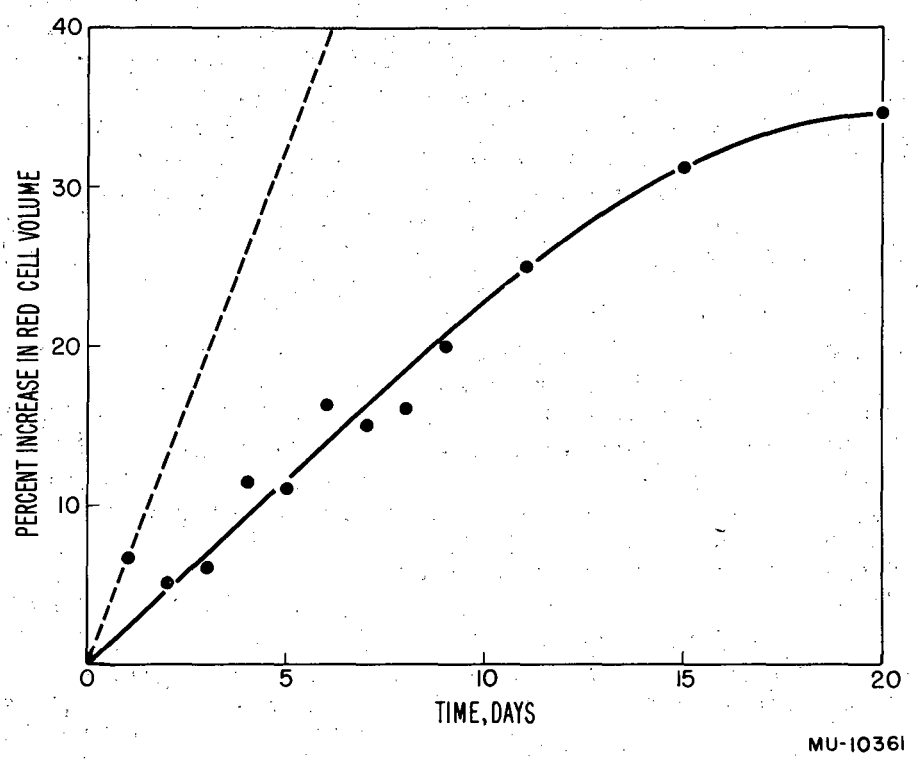

Fig. 7. Percent change in red cell volume in response to an intermittent 6-hour daily exposure to 7.0% oxygen in adult male rats. The broken line indicates the slope of the daily percentage gain in red cell volume of normal rats of 15 days of age.

The inability of the young rat to respond to an hypoxic stimulus is here given as evidence of the high rate of red cell production existing in this young animal. Further, it is postulated that the relative daily red cell production in these young animals is at a maximal or near maximal level and so cannot be stimulated further erythropoietically. Also it is concluded that the relative daily gain in red blood cells in response to hypoxia does not exceed the relative daily gain in red blood cells existing in the young rats.

STUDIES OF RADIOIRON TIME-DISTRIBUTION IN THE RAT AS A FUNCTION OF AGE

The present theory of iron metabolism envisions the pool of iron in the plasma as having a somewhat central position. Although the quantity of iron in this pool remains relatively constant, its level is determined by a balance between rate of removal and rate of supply of iron. Iron enters this plasma pool by assimilation in the gut, by return to it from red cell destruction and from various iron-storage pools in the body. Iron leaving the plasma pool, in the main, is directed to hemoglobin synthesis;[61] but it also enters into the synthesis of various other complex organic substances--myoglobin, cytochromes, peroxidases, and ferritin. Although perhaps to a small extent,[23, 47] a certain amount of iron also leaves the plasma pool by excretion.[37] The mathematical description of the various pool sizes and of the rates of exchange of iron between these pools is the subject of much attention.

Flexner et al. observed after the injection of radioiron that the concentration of radioiron in the plasma decreased exponentially.[36] Huff et al. using the exponential decrease of radioiron in the plasma and the total quantity of iron in the plasma, showed that after the injection of radioiron it was possible to measure the plasma iron turnover.[61] Further, by use of the quantity of the radioiron injected that later appeared in red cells, they showed it was possible to obtain an index of the rate of red cell iron replacement. Wasserman et al. suggest that the plasma clearance of radioiron alone is a sensitive measure of the integrity of the erythropoietic tissue.[117] The values obtained in most cases for red cell iron turnover are higher than expected from consideration of the values obtained for average red cell life. Thus many authors at present consider the existence of a pool of iron which is in a more labile equilibrium with the plasma iron pool than the total storage iron.[34, 48, 74, 104]

Time-distribution studies of radioiron have been carried out in the adult rat after oral administration[8] and after intravenous administration.[62]

The object of this study is to determine the variation with time after injection of the distribution of radioiron in various tissues--plasma, red blood cells, marrow, liver, spleen, muscle, and kidney--at different ages in the rat. The status of erythropoiesis in the rat as a function of age is considered of primary concern.

Methods

Male Long-Evans rats 15, 50, 150, and 250 days of age were used for the study of the distribution of radioiron.

The Fe^{59} was obtained from Oak Ridge as ferric chloride in hydrochloric acid solution. It was neutralized with sodium citrate to a pH of approximately 6, and an aliquot of this was combined just before injection with plasma obtained from a donor rat. Approximately 0.25 microcurie was injected per rat; this gave a counting rate of approximately 100,000 counts per minute on the counter used. The specific activity of the radioiron varied from 1.0 to 4.0 μC per μg of iron, so that approximately 0.06 to 0.25 μg of iron was injected per rat. The total volume of the injection varied from 0.05 ml in the young rats to 0.15 ml in the adult rats. With the use of a specially calibrated syringe, the same volume of the injection mixture as was injected into each animal was put into a volumetric flask and diluted. An aliquot of this was used as a standard and was counted along with the tissues. By use of the proportional difference of this standard from 100,000 counts per minute, the radioactivities of all the tissue samples were corrected to an injection of 100,000 counts per minute. The rats were anesthetized with ether and the injection was made via the saphenous vein. At each of the four ages studied, animals were sacrificed at 13 different times from 15 minutes to 10 days after the time of injection, with the exception of the 15-day-old group, which was carried out to only 5 days postinjection. Three animals were sacrified at each time, making a total of 39 rats per age group.

At the time of sacrifice the rats were anesthetized with ether and the abdomen opened and as much blood as possible was drawn into a heparinized syringe from the vena cava. The animal was then perfused with saline, and a sample each of liver, spleen, marrow, muscle, and kidney was weighed and saved to be counted for Fe^{59} activity. The blood was centrifuged, the plasma removed and the red blood cells washed in saline. An aliquot of the plasma and red cells was saved for counting. The marrow was taken from both femurs and tibias. In the 15-day-old rats, not enough marrow could be gotten from these bones; therefore, as an alternative, these four bones were taken and counted with their bone marrow within. Since chemical iron determinations were done on the same bones, the Fe^{59} activity could be expressed in terms of counts per minute per microgram of iron.

Six rats of each of the age groups studied were used for the determination of the chemical iron in the tissues. These animals were first anesthetized with ether and then injected via the saphenous vein with Fe^{59}-tagged red blood cells obtained from a donor rat. After an allowance of 6 minutes for the cells to mix, the abdomen was opened and as much blood as possible was drawn from the vena cava. The rats were perfused with saline, and a sample each of liver, spleen, marrow, muscle, and kidney was weighed and saved. The Fe^{59} activity was determined in these tissues as well as on an aliquot of blood. These tissues were then wet-ashed and a chemical iron determination carried out according to the method of Ramsey.[94] From the ratio of radioactivity to iron in the blood, and the radioactivity of the tissue, the amount of iron due

to blood that remained in the tissue was calculated. This blood-iron value was then subtracted from the tissue-iron determination so as to give a chemical iron determination free of any contamination due to blood iron. The amount of blood remaining in a tissue after perfusion was considerable, and accounted for from 20% to 40% of the iron determined in the tissue. In the 15-day-old rats, the two femurs and two tibias were taken as the marrow sample.

Results and Discussion

The average data for the distribution of Fe^{59} in the various tissues studied are given in Table VII. Graphical presentation of the data for most of the tissues studied is given in Figs. 8 through 12. Figure 8 is a semilogarithmic plot of the removal of radioiron from the plasma as a function of time for the four different age periods studied. The early values are also plotted on an expanded time scale, and the best line fit through these points, as determined by the method of least squares, in drawn. From the slope of this line, the plasma volume, and the plasma iron concentration, the plasma iron turnover was calculated according to the method described by Huff et al.[61] These values are given in Table VIII in terms of μg of iron per day per animal as well as per 100 g of body weight. The results of these data show that the largest quantity of iron turned over in the plasma pool per unit body weight is seen in rats studied at 15 days of age.

Figure 9 illustrates the increase in radioactivity of the total red cell volume as a function of time after the injection of Fe^{59}. It can be seen that the 50-, 150-, and 250-day-old rats reach a plateau in red cell radioactivity of approximately 72% of the amount of Fe^{59} injected. However, this plateau is reached at approximately 5 days for the 250-day-old rat as compared with 2 days for the 150- and 50-day-old rat. The radioactivity in the red cells of the 15-day-old rats appears to reach a peak of 85% approximately 1 day and then fall to about 80% at 5 days after Fe^{59} injection. It was also observed that in the 15-day-old rats a high concentration of radioactivity appears very early in red cells. According to Finch et al. an estimation of the rate of erythropoiesis may be made from the rapidity with which radioiron enters red cells.[33] At 15 minutes after the injection, approximately 40% of the radioactivity injected was already in red cells in the 15-day-old rat. Since Walsh et al. have observed the incorporation of radioiron in reticulocytes "in vitro",[114] it is suggested that this high uptake of radioiron observed in red cells in the young animal is a reflection of the high percentage of reticulocytes known to be present in rats of this age.[14, 29, 91]

An approximation of the red cell iron replacement rate was obtained by multiplying the plasma iron turnover by the percentage of Fe^{59} seen in red cells. These values, per total animal as well as per 100 g of body weight, are given in Table VIII. Inspection of these data indicates that, relative to body weight, the largest quantity of iron entering the red cell pool per day is to be found in the 15-day-old rat. This value is greater than that in the 150- and 250-day-old rats by a factor of approximately 3.

Table VII

Radioiron time-distribution data in various tissues of male rats of 4 different ages

Age days	15 minutes	30 minutes	1 hour	2 hours	4 hours	6 hours	12 hours	18 hours	1 day	2 days	3 days	5 days	10 days
Plasma - Counts per minute* per total plasma volume													
15	21100	22300	7260	4290	937	655	369	287	388	207	192	135	
50	58700	35000	11900	5000	2280	993	566	773	499	181	176	164	152
150	55800	49100	33100	10100	3580	2340	549	351	307	110	116	76	83
250	61800	44500	28200	12400	3300	2500	548	394	340	158	99	97	68
Red blood cells - Counts per minute per total red cell volume													
15	40800	24900	41400	40000	40200	54000	54300	78200	87600	83700	84000	78200	
50	9700	14800	21100	26400	20700	29600	35800	32300	65400	67400	62300	67700	73000
150	6140	6720	5830	7440	10400	10800	18500	28900	39600	72400	66800	73700	70900
250	5090	7010	9410	6680	13900	10400	26900	22600	30700	55800	57600	68100	72600
Bone marrow - Counts per minute per gram													
15	+ 3600	4010	4090	5180	5180	4500	3260	2210	1520	655	452	302	
50	7270	16700	18400	23600	23500	22700	24800	15500	7640	1940	1500	835	628
150	3760	8540	13400	17300	20400	23600	20400	16800	12700	2410	1470	837	528
250	2770	7390	11600	19100	19750	21000	15100	14300	13900	3125	1350	659	361
Spleen - Counts per minute per total spleen													
15	3530	5025	8470	9830	9700	6850	7650	2910	3140	2450	1715	1835	
50	1050	2680	7180	8480	8090	7985	6200	5360	2575	1520	677	1920	2020
150	462	668	1310	3860	3940	2760	2840	2500	2250	1600	1510	1270	1450
250	217	534	1590	4030	3360	3010	3560	2830	1900	1340	1020	985	1070
Liver - Counts per minute per total liver													
15	12600	15500	14500	16800	13200	16200	12800	13400	16800	10300	6650	5070	
50	5190	12900	14800	12900	14800	14100	12900	12000	10700	6510	6125	5260	3040
150	2270	4030	6070	10100	10100	12500	11400	11600	11500	9400	8190	8545	7305
250	1990	5130	7980	8800	7800	10300	8380	10400	10500	10100	10500	7940	7530
Muscle - Counts per minute per gram													
15	407	469	518	692	468	621	700	675	525	598	582	412	
50	71	66	63	66	64	70	92	112	103	100	93	110	108
150	23	20	21	17	13	23	23	18	21	25	24	30	26
250	17	17	16	14	13	15	18	16	17	20	22	19	24
Kidney - Counts per minute per total kidney													
15	1220	1420	1210	1420	1260	1480	1510	1340	1530	1460	1190	1130	
50	1310	1020	768	676	726	730	900	824	720	726	636	616	624
150	1210	1070	1130	732	656	802	832	692	644	754	712	722	598
250	1330	1000	1030	786	664	704	708	792	708	764	796	624	668

* Radioactivity of all tissues corrected to an injection of 100000 Counts per minute.

+ Counts per minute for both tibias and both femurs for 15 day old rats only.

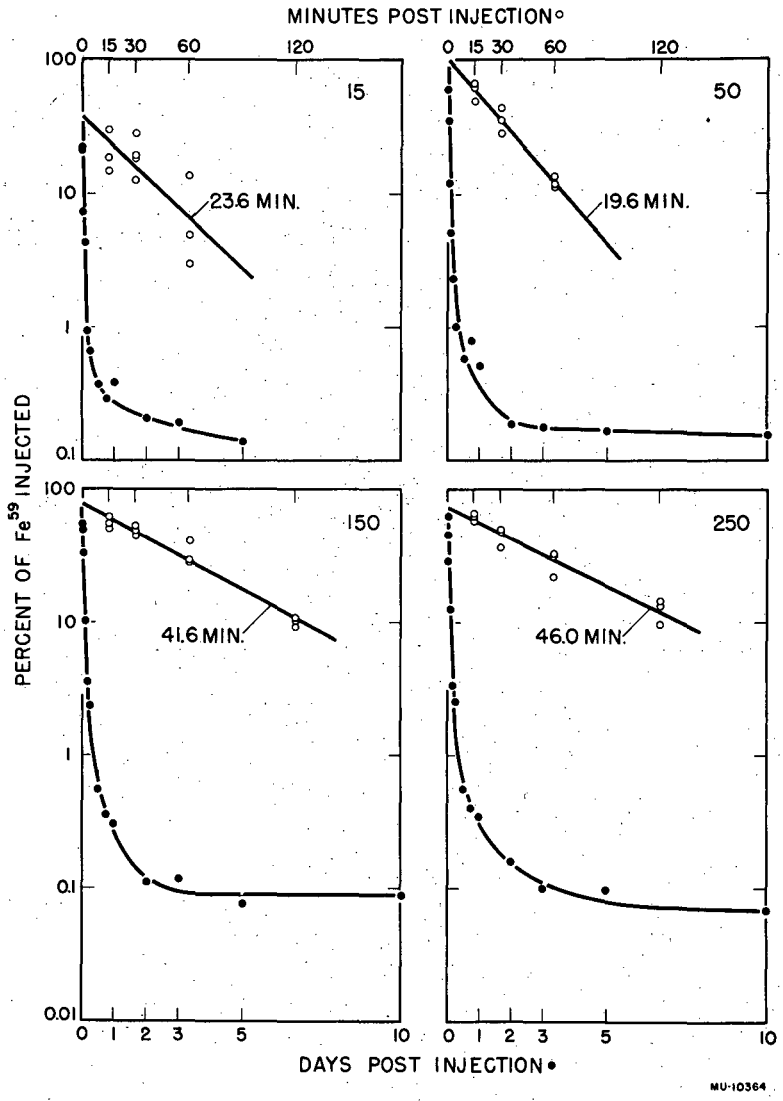

Fig. 8. Radioactivity in total plasma volume of rats 15, 50, 150, and 250 days of age as a function of time after the injection of Fe^{59}. The open circles indicate the early points, using the time scale above, and the closed circles refer to the time scale given below.

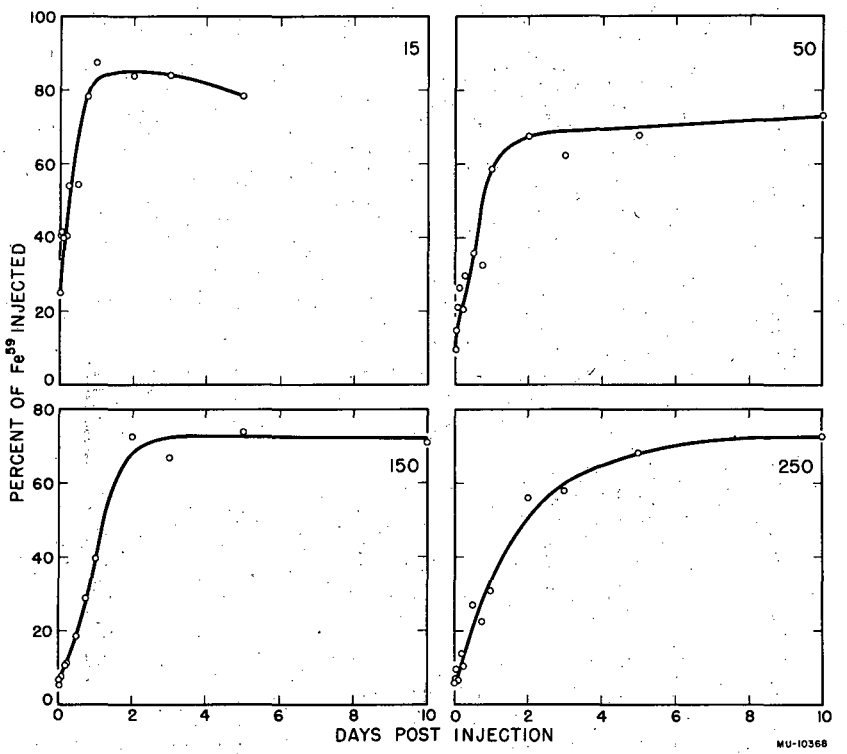

Fig. 9. Fe^{59} radioactivity in the total red cell volume of rats 15, 50, 150, and 250 days of age as a function of time after injection.

Table VIII

Plasma and red cell iron turnover data at various ages in the male rat

Age	Wt.	Plasma iron	Plasma volume	Total plasma iron	Plasma iron clearance rate ($T_{\frac{1}{2}}$)	Plasma iron turnover*		Fraction radioiron in red cells	Red cell iron turnover	
days	(g)	(μg /ml)	(ml)	(μg)	(minutes)	(μg / day)	(μg /day/ 100 g body wt.)	(%)	(μg / day)	(μg./day/ 100 g body wt.)
15	33.5	1.13	1.81	2.05	23.6	86.7	259	85	73.9	221
50	165	1.20	5.87	7.04	19.6	358	217	72	258	156
150	348	1.74	8.56	14.9	41.6	357	103	72	257	73.8
250	409	1.75	9.82	17.2	46.0	373	91.2	72	269	65.8

* Plasma iron turnover = $\frac{0.693 \text{ Total plasma iron}}{T_{\frac{1}{2}}}$

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In Fig. 10 a plot is given of the radioactivity per μg of iron in the bone marrow as a function of time after injection. This was obtained simply by the division of the radioactivity per g of marrow by the quantity of iron determined per g of marrow for each age studied. The average data for the concentration of iron in the tissues studied is given in Table IX. In the 15-day-old rats, the total radioactivity obtained for both tibias and for both femurs was divided by the total quantity of iron determined in these same bones. Inspection of these curves shows that the radioactivity of the marrow for the 15-day-old rats rises to its peak and falls faster than in the 150- and 250-day-old rats. It is seen that in the 15-day-old rats even the early points, taken 15 minutes after injection, are approximately two thirds of the peak value obtained; whereas a comparable value is reached at somewhere between 1 and 2 hours after injection for the 150- and 250-day-old rats. The activity in the marrow of the 15-day-old rats falls to half the peak value at approximately 15 hours, whereas in the 150- and 250-day-old rats this occurs at approximately 24 hours after injection. The peak value in the 250-day-old rats corresponds to approximately 21,000 counts per minute in terms of radioactivity per g of marrow. If all the radioactivity is totaled in the other tissues, at this time, and if the remainder is assumed to be in the marrow, then a maximum figure for marrow volume can be obtained. This would assume that the sample of marrow obtained was a representative sample of marrow in the animal. The marrow volume calculated in this manner is approximately 3.2 g in the 250-day-old rat or 0.8 g per 100 g body weight. The same value of 0.8 g of marrow per 100 g of body weight is obtained also for the 150-day-old rats; however, this calculation for the 50-day-old rat gives a value of 1.2 g of marrow per 100 g body weight. Unfortunately, no value could be obtained for marrow volume in the 15-day-old rats, since it was difficult to obtain an accurately weighed sample of this tissue in this animal.

Figure 11 is a plot of the total spleen radioactivity for the four age periods studied. The spleen radioactivity rises to 4% of the injected Fe^{59} at 2 to 6 hours after injection in the 250-day-old rats, and falls to and remains at a level of approximately 1% 3 days postinjection. The curve for the 150-day-old rats is similar, except that it levels at approximately 1.5% of the injected radioiron. The 15-day-old rat shows a spleen curve very similar to the curve obtained in the marrow of this animal; not only in time relationships, but also when the radioactivity per microgram of iron in the spleen is calculated for this animal, it is seen that at the peak value the concentration of radioactivity per unit of iron is approximately the same for both the marrow and spleen. This would seem to indicate that all of the iron in the spleen of these young rats is involved in erythropoiesis in this organ. The spleen radioactivity in the 50-day-old rat also indicates that a certain amount of erythropoiesis is occurring in the spleen of this animal; the radioactivity per microgram of iron in the spleen at the peak is approximately half the peak value in the marrow. An approximation of the relative amounts of erythropoiesis occurring in the spleen relative to total spleen and marrow can be obtained if all of the radioactivity in the tissues analyzed, with the exception of the marrow and the spleen, is summed, and if all the rest of the activity is assumed to be involved in erythropoiesis in either the marrow or spleen. This is assuming that there is no loss of the radioiron injected at 4 to 6 hours postinjection when the spleen and marrow activity are highest. This value comes out to approximately

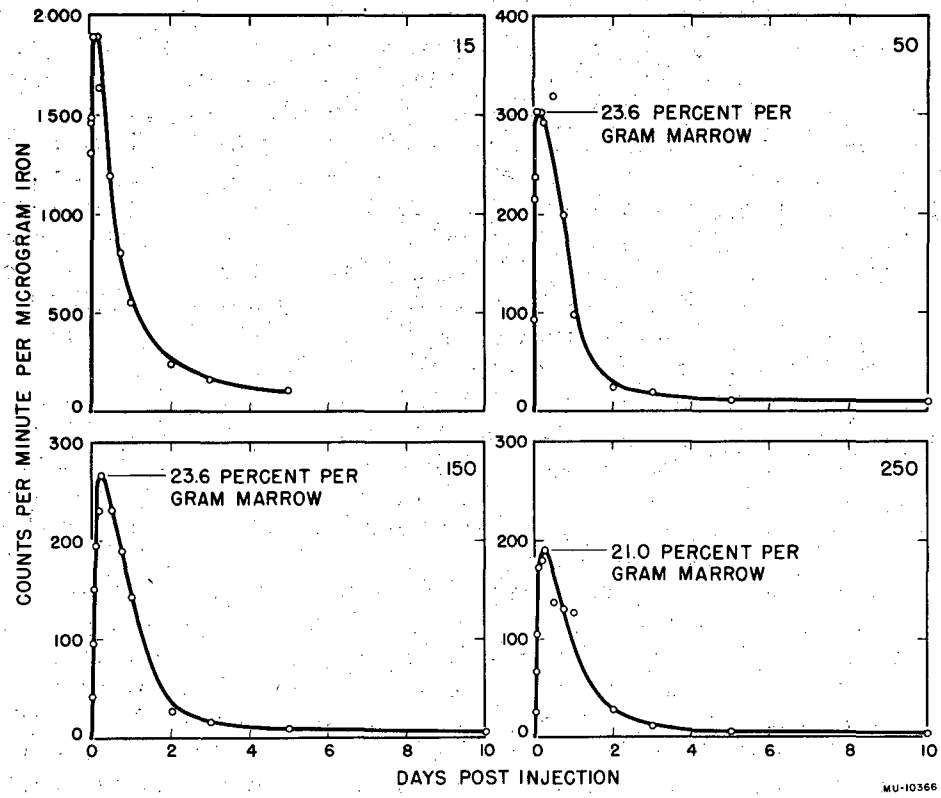

Fig. 10. Fe^{59} radioactivity in marrow in terms of counts per minute per μg of marrow iron for rats 15, 50, 150, and 250 days of age. The figures to the right of the peak values indicate the percent of the injected Fe^{59} per g of marrow at the highest point in marrow.

Table IX

Average total iron data of tissues of male rats of different ages

Age, days	15	50	150	250
Plasma, ug / ml	1.13 (12)*	1.20 (20)	1.74 (41)	1.75 (35)
Blood, ug / ml	198	388	476	481
Red cells, ug / ml	808	1013	1010	978
Bone marrow, ug / g	2.74 ‡	77.9	88.5	110
Spleen, ug / g	37.2	64.0	182	476
Liver, ug / g	15.0	19.4	18.9	52.3
Muscle, ug / g	6.5	11.6	7.0	14.0
Kidney, ug / g	9.4	23.5	22.7	30.2
Relative organ weights (g / 100 g body weight)				
Spleen	0.408	0.426	0.269	0.201
Liver	3.98	5.40	3.90	3.60
Kidney	1.30	0.846	0.720	0.652

* All values are an average of six determinations, unless otherwise indicated by number in parenthesis.

‡ The value given for bone marrow, of the 15-day-old rat only, is in terms of micrograms of iron per both tibias and both femurs.

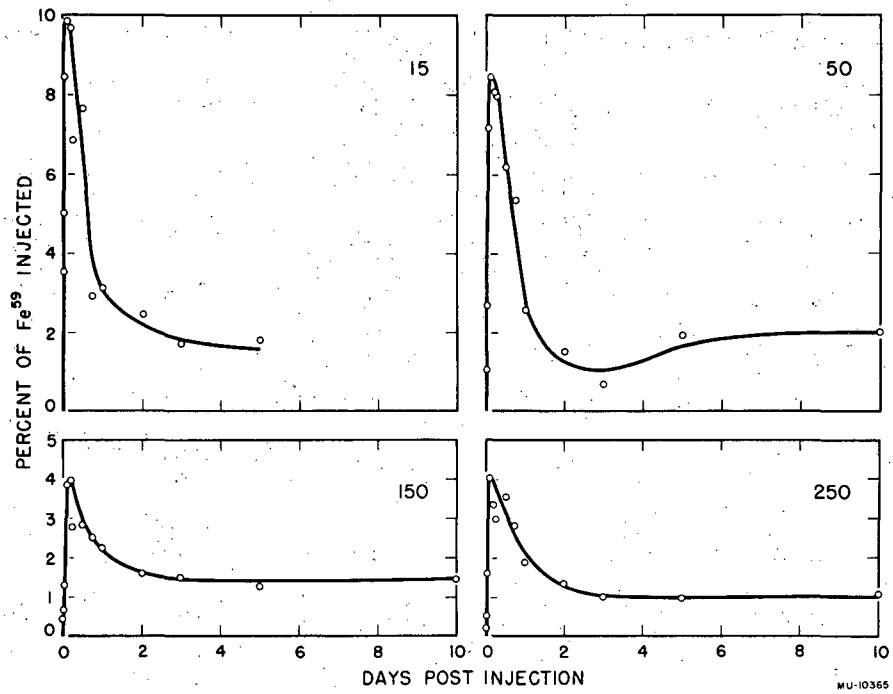

Fig. 11. Fe^{59} radioactivity in the total spleen of rats of 15, 50, 150, and 250 days of age as a function of time after the injection.

30% for the 15-day-old rat; that is, approximately 30% of the erythropoiesis in this animal occurs in the spleen. It is interesting to note in this respect that Kindred observed in the 15-day-old rat that 22% of the red cells are produced in the spleen.[79] Also, it was noted by Huff et al., in adult rats receiving total body irradiation with lead protection of their spleens, that evidence of erythropoiesis was observed in the spleen; and further that the amount of radioiron which later appeared in red cells was approximately 30% of the normal control value.[62] Approximately 16% of the red cells in the 50-day-old rat are produced in the spleen. This is reduced to 5% for the 150- and 250-day-old rats.

In Fig. 12 are presented the data for the radioactivity in the total liver of the four age groups studied. In this tissue also the Fe^{59} activity rises early and then falls to and remains at a level of approximately 8% in the 150- and 250-day-old rats. The Fe^{59} radioactivity in the younger rats studied appears to rise to a higher level and to fall to a lower level than in the 150- and 250-day-old rats.

In Fig. 5 was presented a curve for the growth of the total red cell volume with age, as well as a curve for the gain in total red cell volume per day as a function of age. This latter curve indicates, as was pointed out earlier, the daily production of red cells in excess of that production which is required to maintain the red cell volume not withstanding continuing red cell destruction. The average red cell life in the rat has been measured by various authors, with values ranging between 45 and 60 days.[16, 37, 39] If a value of 50 days is assumed, and if it is assumed to be constant throughout the life of the rat, then 1/50 of the total red cell volume must be produced per day to maintain the total red cell volume. If 1/50 of the total red cell volume existing on a particular day is added to the gain in red cell volume on that day, and if this is carried out for the age range covered, then a curve for the total daily red cell production as a function of age is obtained. Such a curve is presented in Fig. 13. It would appear from this curve that the daily production of red cells rises rapidly in the rat to about 40 days of age, and then remains relatively constant out to 260 days of age. This would indicate a production of approximately 0.18 ml of red cells per day per total animal from 40 to 260 days of age. Inspection of Table VIII shows that the value for red cell iron turnover per day calculated for rats of 50, 150, and 250 days of age are quite close, varying between 257 and 269 μg of iron per day. These values are somewhat higher than would be expected from Fig. 13, but it would seem significant that they agree at least in that they indicate a constant production of red cells regardless of the age of the animal from 50 to 250 days of age.

Also shown in Fig. 13 is a curve for the volume of red cells produced per day per 100 g of body weight as a function of age in the rat. At 15 to 20 days of age this curve would indicate that approximately 0.12 ml of red cells is produced per day per 100 g of rat. This is reduced to approximately 0.04 ml of red cells produced per day per 100 g of rat at 150 and 250 days of age. This would suggest that relative to body weight, the red cell produced in 15-day-old rats is greater than in animals of 150 to 250 days of age by a factor of approximately three. When the values, as given in Table VIII for red cell iron turnover per unit

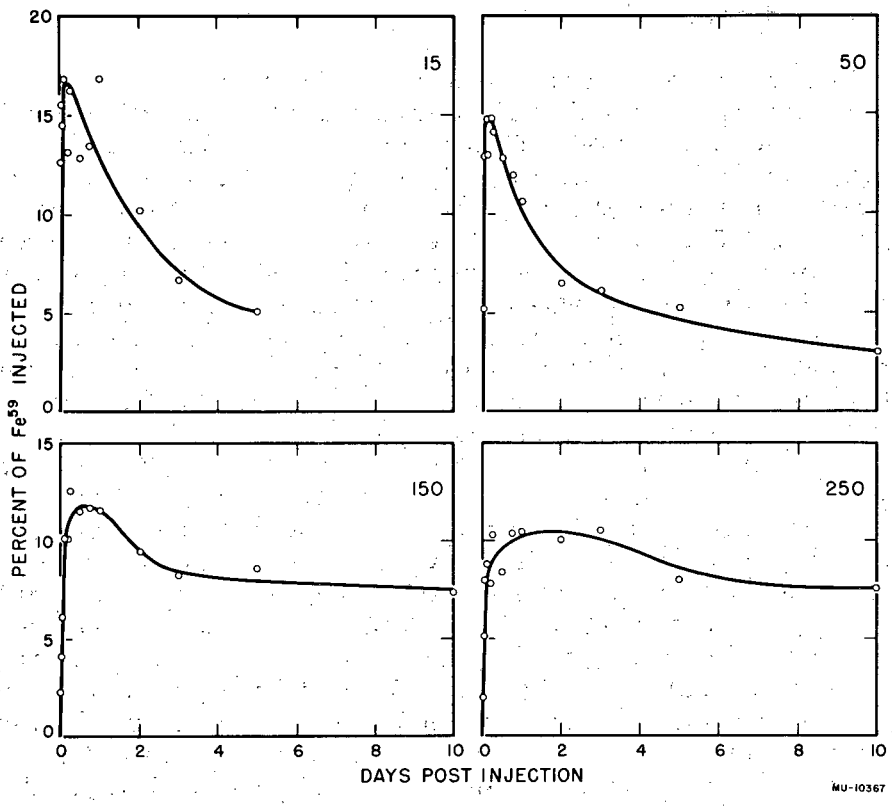

Fig. 12. Fe^{59} radioactivity in the total liver of rats of 15, 50, 150, and 250 days of age as a function of time after the injection.

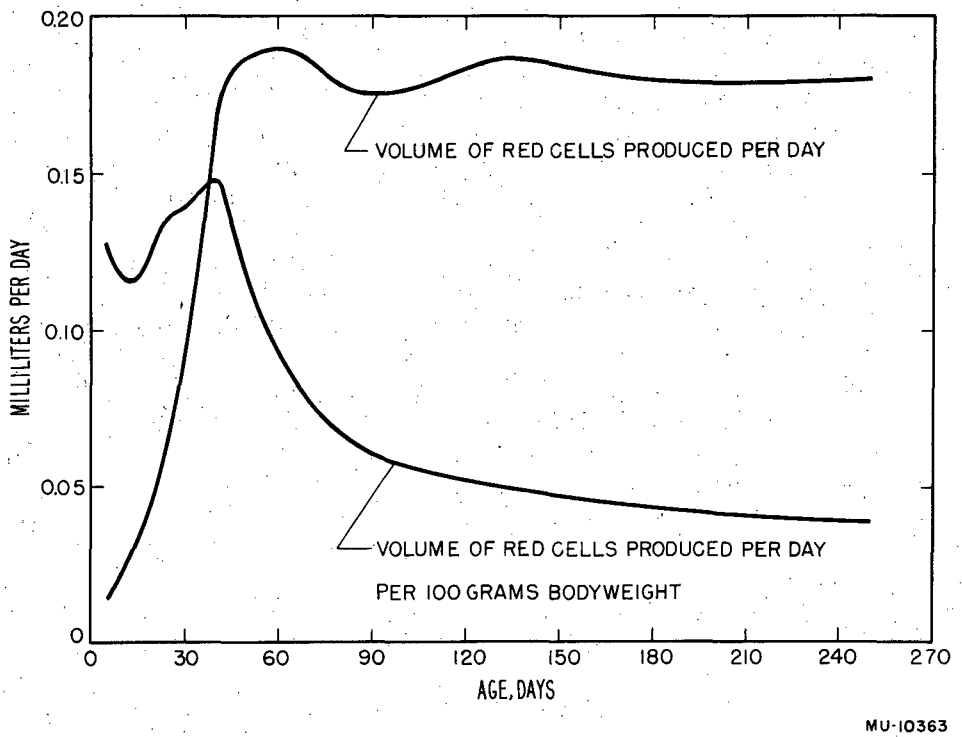

Fig. 13. Daily production of red blood cells per rat, and per 100 grams of rat, as a function of age, assuming an average red cell life of 50 days.

body weight, are compared with the curve in Fig. 13 for red cell production relative to body weight, it is seen again that relative to body weight, the 15-day-old rat directs approximately three times as much iron to the red cell pool as does the rat of 150 or 250 days of age. It is realized that some assumptions are involved in such a comparison; however, it is gratifying to find agreement by two completely separate techniques of study.

Summary

The distribution of radioiron with time has been studied in the plasma, red cells, liver, spleen, marrow, muscle, and kidney in rats 15, 50, 150, and 250 days of age. The plasma clearance of Fe^{59} is very rapid in the two youngest ages studied and much slower in the 150- and 200-day-old rats. Fe^{59} enters the red cell most rapidly in the 15- and most slowly in the 250-day-old rats. The radioactivity in the marrow also indicates a more rapid entry and exist in this tissue in the young rat. Comparison of the time relationships in the Fe^{59} distribution in the spleen and marrow as well as the Fe^{59} activity per μg of iron in these tissues suggests that the iron in the spleen in the 15-day-old rat is involved in erythropoiesis to the same extent as the marrow is. Approximately 30% of the erythropoiesis occurring in the rat at this time occurs in the spleen. Erythropoiesis is also suggested in the spleen of the 50-day-old rat, although to a somewhat lesser degree.

Plasma and red cell iron turnovers were calculated. Approximately equal values for total red cell iron turnover were obtained for rats of 50, 150, and 250 days of age regardless of wide differences in body weight. The red cell iron turnover relative to body weight was highest in the 15-day-old rats; in them it was approximately three times that calculated for the 150- and 250-day-old rats. When these values for red cell iron turnover were compared with the values obtained by analysis of the red cell volume data for red cell production, good agreement was obtained with the exception that the values obtained for red cell iron turnover were somewhat higher than expected.

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List of Abbreviations Used in Tables and Figures

BV	blood volume
g	gram
Hb	hemoglobin
Hct	hematocrit
ml	milliliter
No	number
PV	plasma volume
RCV	red cell volume
SD	standard deviation
T1/2	half time
TBV	total blood volume
THb	total hemoglobin
TPV	total plasma volume
TRCV	total red cell volume
µg	microgram
wt	weight