

**Iron Clearance and Utilization in Rats**

**Using a Direct Method of Counting Fe<sup>59</sup>**

**Daniel Nathans**

**Senior, Washington University School of Medicine**

## Introduction

The rate of disappearance of radioiron from the serum (clearance) and its rate of appearance in circulating red blood cells (utilization) have for many years been used as indices of red cell production and release from bone marrow. Since red cells incubated in vitro do not exchange iron with the medium, the injected radioiron appearing in red cells in vivo must be in newly synthesized hemoglobin. ( ) Therefore the iron utilization reflects the rate of hemoglobin production, though the exact relationship remains to be worked out. The relation of iron clearance to red cell production is even less clear. Clearance does not change uniformly with a given change in utilization. ( ) Factors other than bone marrow extraction of iron are concerned, e.g., the liver has been shown to take up injected radioiron, at least temporarily. ( ) However Dubach, Moore, and Minnich have shown in man and in dogs that injected iron enters a relatively small highly labile iron pool which is apparently used preferentially for hemoglobin synthesis. ( )

It would be useful to have a simple method of determining radioiron clearance and utilization in a large number of small laboratory animals such as the rat, in order to determine the effect of various drugs, procedures and pathologic states on erythropoiesis. Such a method would require 1) that a small amount of radioiron be needed, 2) that small blood or serum samples have sufficient activity to measure, and 3) a simple method of counting radioiron in these small samples.

The usual method of counting  $\text{Fe}^{59}$  in biologic material has involved ashing the sample, taking up the iron as a salt, adding carrier iron and electroplating this solution onto a planchet, which is then counted with

a Geiger tube.( ) This method depends on minimizing the self-absorption of the low-energy  $\text{Fe}^{59}$  beta rays.( ) It has been used to determine iron utilization in rats by Copp & Greenberg,( ) who sacrificed animals at varying times and therefore were able to use large volumes of blood and by Hennessey & Huff( ) who used 0.5cc volumes of blood from the same animal at daily intervals. However, this method is felt to be cumbersome for volumes of blood or serum in the range of 50 to 100 microliters, especially if large numbers of animals are to be used.

A more recently reported method of counting  $\text{Fe}^{59}$  makes use of the penetrating gamma ray emission of the isotope, which is counted with a scintillation counter.( ) No prior chemical treatment of the sample of blood or serum is required since self-absorption is not a factor. Although this method is simple, it was found too insensitive in this study when small amounts of radioiron were injected and small samples of blood and serum were used.

#### Method

From a theoretical standpoint, given radioiron of sufficient activity, one should be able to maximize sample thickness so that only the top layer of the sample is counted, or in other words so that a constant fraction of the radiation is absorbed by the sample.( ) This would result in counts which are proportional to the amount of radioiron in the sample. In attempting to do this it was found by trial and error that when samples of water, hemolysed blood or serum, with varying amounts of radioiron, are pipetted onto filter paper discs glued to planchets and then oven-dried, the solution spreads out evenly over the filter paper and the measured counts are proportional to the amount of radioiron in the sample (Figs 1 & 2 & table I). It will be noted that the best straight line is almost identical whether the medium is serum or hemolysed blood, again

indicating that sample thickness is probably maximal.

To test the effect of sample configuration on the measured counts a water solution of  $\text{Fe}^{59}$  was pipetted onto filter paper of varying shape and size. The results are presented in table II. It will be noted that rather marked changes in configuration and size of the filter paper resulted in only small if any changes in the measured counts using the flow counter as described below.

In practice, Whatman #54 filter paper discs, measuring approximately 20 mm. in diameter, are glued to copper planchets of 22 mm. diameter with a hydrophobic glue or cement (e.g. Rubber cement). When 50 to 100 microliters of the  $\text{Fe}^{59}$  containing blood (1 part blood:1 part 0.15N NaOH) is pipetted onto the filter paper using a constriction pipette\* ( ). The solution must spread out evenly over the filter paper. The discs are dried in an oven at  $120^{\circ}\text{C}$  for 10 minutes, allowed to cool, and counted. The instrument used in this work is a flow counter, using a 90% argon 10% methane gas mixture, attached to a "Tracerlab" autoscaler with a Geiger-Mueller tube.

### Radioiron Clearance and Utilization

#### Procedure

Rats were injected I.V. via tail vein with 0.5cc of the  $\text{Fe}^{59}\text{SO}_4$  solution containing less than 1 gamma of iron\*\*. Tail samples were

\*Rat hemoglobin is only slightly soluble in water and must therefore be dissolved in dilute alkali. If the alkali is much greater than 0.08 N final concentration a dark brown viscid gel forms which is difficult or impossible to pipette, and this will not spread evenly on the filter paper. Equal parts of blood and 0.15 N NaOH results in an easily handled homogeneous solution after 5 minutes standing at room temperature. It may safely be heated to  $50^{\circ}\text{C}$  to hasten solution, but high temperatures result in precipitation of the altered hemoglobin.

\*\*Since prior determination of the unbound serum iron-binding protein in a single rat revealed a value equivalent to 370 gamma % iron, the injected dose of iron probably did not exceed the iron-binding capacity and was therefore a true tracer dose. A micro-adaptation of the method of Rath & Finch was used( ), the samples being read at 460mu since rat serum, unlike human serum, has a negligible absorption at this wave length.

collected at varying time intervals thereafter, either in 2 mm glass tubes (for serum) or in test tubes containing a small amount of heparin powder (for blood). The serum or whole blood was then prepared for counting as detailed above. Most of the samples in this study were in the range 70 to 400 cts/min. (10 to 100 times the background count). The hematocrits of the blood samples were determined, generally in capillary tubes.

It had been previously determined that after 5 to 10 hours there is little measurable activity in the serum of rats given an I.V. dose of  $Fe^{59}$ , the exact time depending on the dose. Nearly all the activity after this time is in the red cells. Whenever red cell  $Fe^{59}$  was determined at less than 5 hours the centrifuged red cells were washed three times with normal saline.

#### Calculations

In calculating percent utilization of injected  $Fe^{59}$  the blood volume was assumed to be 4.59 cc/100 gm. as determined by Berlin et al using isotopically tagged red cells ( ). Hennessey and Huff give the following formula for calculating the percent of injected radioiron in the red cells, using Berlin's figure ( ):

$$\frac{\text{Animal weight} \times 4.59 \times \text{Hematocrit} \times \text{CPM/cc of red cell sample} \times 100}{100 \quad \text{Total CPM injected}}$$

However, since "cc. of red cell sample" is determined by multiplying the volume of the blood sample by the hematocrit, the hematocrit values cancel out in the formula, and it is therefore not necessary to consider the hematocrit in the calculation. In the present study the above formula was used (without the hematocrit value), although this method disregards the effect of sampling blood loss on per cent iron utilization.

Prior determination of utilization curves in rats both by us and by Hennessey and Huff ( ) showed maximal utilization of intravenously injected iron after 3 days. For this reason the utilization experiments were not

carried beyond 72 hours.

#### Results in Normal Adult Rats:

Simultaneous iron clearance and utilization curves were completed in two normal adult female rats weighing 200 and 220 gm., respectively. Iron clearance alone was determined in a third animal weighing 200 gm. The injected dose measured 130,000 cts/min. in each case. The results are plotted in figs. 3 and 4 and tables III and IV. The graphically determined initial half-time for  $\text{Fe}^{59}$  clearance from the serum are 86, 90, and 88 minutes, respectively. Maximum iron utilizations are 69.5 and 57%, respectively.

#### Effect of Splenectomy:

Clinical hypersplenism states have raised many questions concerning the function of the normal mammalian spleen. Although it has been known for some time that increased destruction of red cells can result from splenic dysfunction, it has only recently been emphasized that deranged splenic function can result in decreased red cell production as determined by radioiron utilization, which increases after splenectomy ( ). The following hematologic changes have been reported to occur following splenectomy in certain normal mammals: increased circulating white blood cells, reticulocytes, and platelets; the appearance of nucleated red cells and Howell-Jolly bodies in the circulation; hyperplasia of the bone marrow; and in some cases polycythemia.

The present study was undertaken to determine the effect of the spleen of normal rats on erythropoiesis as determined by radioiron clearance and utilization measurements. A group of young female rats weighing about 150 gm. each was splenectomized, and a second control group of the same stock and weight was operated on and a piece of omentum removed. Each group of animals was given subcutaneous aureomycin (6 mg twice a day for 7 days) to prevent possible Bartonella muris anemia in the splenectomized

animals and to eradicate possible latent Bartonellosis in the control group ( ). None of the animals developed hemolytic anemia.

The results of the clearance studies are presented in figs. 5 and 6 and table IV. Figs. 5 and 6 show the serum clearance curves plotted on semilogarithmic paper. As seen from the graph the clearance rates are practically identical in the normal and splenectomized groups. The actual data and the graphically determined half-time for the initial period are presented in table V.

The iron utilization curves are shown in figs. 7 and 8 and the actual data and maximal utilizations in table VI. As seen in the figures and in the table there is a wide range of values in both groups and the two groups overlap. One possible explanation for this scatter is the varying blood loss which may occur with the sampling procedure, in addition to the variation in initial hematocrit. With these reservations, the data suggest that there is no marked change in iron utilization following splenectomy in the normal rat.

#### Discussion

The error of the method for direct counting of  $Fe^{59}$  described here is higher than that reported for the ashing and electroplating procedure generally employed. (Hennessey and Huff report a "counting error" of 1% ( ) and Copp and Greenberg report iron recovery of % ( ). I am unaware of any reports on the errors involved in the use of the scintillation counter for counting  $Fe^{59}$ . The reproducibility of counts of similar samples by direct counting is generally within 6%, most of those outside these limits showing erratic counts on the same sample (see table I). This error might be reduced if longer counting periods are employed.

The use of the direct method of counting  $Fe^{59}$  in the serum and blood of small laboratory animals has been illustrated by its application to the

investigation of normal splenic function. The results indicate that there is no difference in radioiron clearance in normal control and splenectomized rats and probably no difference in utilization. However under certain marrow stimulating conditions, e.g., anoxia, blood loss or cobalt administration, erythropoiesis may be affected by the normal spleen and this seems worth investigating. The present method could be applied to many other problems of iron metabolism and bone marrow function. For example the rate at which non-hemoglobin iron is converted to hemoglobin iron in the marrow might be determined by measuring the amount of injected radioiron found in various chemical fractions of bone marrow. Recently Palmer has succeeded in producing "hypersplenism" in rats given methyl cellulose for several weeks ( ), and it would be interesting to look for changes in iron clearance and utilization in these animals. Also it is now possible to produce chronic renal failure in rats in a period of a few weeks ( ), and the effect of this state on the handling of radioiron could be readily investigated.

The procedure outlined above could be further simplified so that only maximal utilization is determined (i.e., one blood sample counted after 72-96 hours). By taking these samples, two early serum samples and one later blood sample, the clearance rate and maximal utilization of iron can be determined with minimal trauma to the animal and a total of about 0.30 ml of blood. With an additional 0.1 cc of serum, iron and iron-binding capacity could be determined. If desired, the life span of the red cells can be measured by following the rate of disappearance of radioiron from the red cells.

#### Conclusions

1) A method for direct counting of radioiron( $\text{Fe}^{59}$ ) in the serum and blood of small animals has been described. It depends on maximizing sample thickness so that ~~counts~~ counts are proportional to the amount of  $\text{Fe}^{59}$

in the sample.

2) This method was applied to the study of iron-clearance and utilization in individual normal adult rats and in a control and splenectomized group of younger animals. The initial clearance half-time in adult female rats was found to be 86 to 90 minutes and the maximum utilization about 65%. The clearance half-times were essentially the same in splenectomized and control rats (50-70 minutes). Iron utilization was variable in each group with overlapping of values between the two groups. These data suggest that under normal conditions the spleen of the rat has no marked effect on erythropoiesis.

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