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The Effect of Transferrin Saturation on Internal Iron Exchange[1] (42387)

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Abstract. Radioiron was introduced into the intestinal lumen to evaluate absorption, injected as nonviable red cells to evaluate reticuloendothelial (RE) processing of iron, and injected as hemoglobin to evaluate hepatocyte iron processing. Redistribution of iron through the plasma was evaluated in control animals and animals whose transferrin was saturated by iron infusion. Radioiron introduced into the lumen of the gut as ferrous sulfate and as transferrin-bound iron was absorbed about half as well in iron-infused animals, and absorbed iron was localized in the liver. The similar absorption of transferrin-bound iron suggested that absorption of ferrous iron occurred via the mucosal cell and did not enter by diffusion. The decrease in absorption was associated with an increase in mucosal iron and ferritin content produced by the iron infusion. An inverse relationship ($r = -0.895$) was shown between mucosal ferritin iron and absorption. When iron was injected as nonviable red cells, it was deposited predominantly in reticuloendothelial cells of the spleen. Return of this radioiron to the plasma was only 6% of that in control animals. While there was some movement of iron from spleen to liver, this could be accounted for by intravascular hemolysis. Injected hemoglobin tagged with radioiron was for the most part taken up and held by the liver. Some 13% initially localized in the marrow in iron-infused animals was shown to be storage iron unavailable for hemoglobin synthesis. These studies demonstrate the hepatic trapping of absorbed iron and the inability of either RE cell or hepatocyte to release iron in the transferrin-saturated animal.

The internal movement of iron is mediated by the plasma protein, transferrin. Iron delivery to tissues involves the initial complexing of iron-loaded transferrin iron with membrane receptors and the subsequent internalization of the complex with release of the iron (1). Much less is known about the process whereby iron is procured by plasma transferrin. There is evidence that iron atoms react at random with the open iron-binding sites of transferrin and consequently that one atom of iron is taken up at a time (2, 3). This could occur through either the release of iron from the membrane of the iron-donating cell into the plasma and subsequent uptake by circulating transferrin, or it could require direct interaction between unsaturated transferrin and the membrane iron of donor cells. In this study, the effect of a blockade of transferrin-binding sites on the entrance of iron into the plasma was evaluated.

Materials and Methods. Normal male Sprague–Dawley rats, 8 to 12 weeks old, weighing 195 to 260 g, were employed. The rats were maintained on a standard Purina rat chow diet. Two to four days before the experiment a PE-50 polyethylene tube was inserted into the superior vena cava of rats under ether or innovar anesthesia (4). The night before the experiment rats were fasted but had free access to water. On the day of the experiment, one rat received an infusion of iron through the jugular catheter to saturate plasma transferrin, while a control animal was studied without iron infusion. With the exception of the iron infusion, the experimental procedure was the same for both control and iron-injected animals.

The iron infusion began with the introduction over a period of 10 min of 40 μg of iron as ferrous ammonium sulfate (pH 1.8). This was followed by a constant infusion of 150 μg/hr for 3 hr before the administration of the labeled compound and was continued until

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the end of the experiment 2 or 3 hr later. The criterion for adequacy of the iron infusion was a transferrin saturation of >95% at the termination of the study.

At the end of each study, anesthetized rats were exsanguinated through the abdominal aorta and perfused with 20 ml of cold saline. The liver, spleen, kidneys along with 1-ml aliquots of whole blood and plasma were counted for radioactivity in a gamma counter. Skeletal activity was estimated by multiplying the counts of one femur by 13 (5). The blood, plasma, and red cell activities were obtained by counts of whole blood and plasma, corrected for an estimated blood volume of 6 ml/100 g body weight. Absorbed activity was calculated from the combined activity of liver, spleen, skeleton, and blood. When heat-damaged red cells or a hemoglobin–haptoglobin complex was used, the sum of the ^{59}Fe activity in red cells, plasma, bone marrow, liver, spleen, kidneys, and urine was assumed to represent 100% of the injected activity. These selected tissues accounted for most of the body radioactivity, and counts in a small body counter (Armac Packard Model 447) with the gut excluded gave comparable results for total body activity. However, tissue counts were considered preferable because of their greater reproducibility.

Absorption studies. Absorption was measured by introducing into the gut lumen 15 μg of ^{59}Fe (0.5 μCi) as either $FeSO_4$ or diferric transferrin. The purified rat transferrin was prepared as previously described (6). The abdomen of the anesthetized animal was opened and a ligature placed at the pylorus to prevent reflux of injected iron. The tagged compound was introduced through the ligature which was then tightened. The abdominal wound was closed by clips and the animal kept in a temperature-controlled environment until sacrifice 2 or 3 hr later.

At sacrifice a 30-cm segment of the gut, starting from the pylorus, was removed, washed with 60 ml of pH 7.4 phosphate-buffered saline, and the mucosa scraped off with a glass slide. After addition of 4 ml of phosphate-buffered saline, a mucosal homogenate was prepared with a Teflon homogenizer. Nonheme iron content of the mucosa was determined with the method of Kaldor (7). Ferritin iron was determined after overnight incubation in 3 *N* HCl at 37°C using a colorimetric method (8).

In additional studies evaluating the contribution of infused and absorbed iron to mucosal iron content, ^{59}Fe transferrin was injected into the gut as described above. At the same time the infusion solution was labeled with ^{55}Fe. After 1 hr the gut segment was removed and washed and villous cells were exfoliated from an everted loop by vibration (9). Relative activities of ^{55}Fe and ^{59}Fe were determined. From the specific activity of infused and absorbed iron, contributions of each to mucosal iron were determined.

Nonviable red cells. For studies of reticuloendothelial behavior, radioiron-tagged nonviable red cells were employed. Radioiron tagging of red cells *in vivo* was accomplished as previously described (10) and resulted in a specific activity of 0.1 to 0.2 μCi/mg hemoglobin. Red cells were heat damaged by first washing with phosphate-buffered saline, resuspending the cells in ACD (formula B), and heating in a water bath at 41.1°C for 9 min (11). They were then injected through the tail vein within 1 hr of their preparation in an amount equivalent to 3.2 ± 0.2 mg of hemoglobin. Red cell disappearance from circulation was simultaneously monitored in a normal rat, and the cells were considered satisfactory for study when the $T_{1/2}$ disappearance rate was between 10 and 25 min. Animals were sacrificed 3 hr after the injection of heat-damaged red cells.

In some studies of redistribution of red cell iron, in addition to the iron infusion, 10 mg of ^{55}Fe-tagged hemoglobin was injected 30 min before the heat-damaged red cell injection, followed by a continuous infusion of 30 mg/hr until the time of sacrifice. This amount of hemoglobin more than saturated circulating haptoglobin. The purpose was to divert a larger portion of intravascular hemoglobin derived from heat-damaged red cells into the urine. The distribution of the ^{59}Fe injected as heat-damaged red cells and the ^{55}Fe as hemoglobin was determined at 30 min and 3 hr. In these experiments, urine as well as selected tissues were counted for ^{55}Fe and ^{59}Fe activity. In control studies it was shown that iron and hemoglobin infusions had no effect on the initial tissue uptake of heat-damaged red cells. Thus, spleen and liver activity 30 min after injection

TABLE I. EFFECT OF iv IRON INFUSION ON IRON ABSORPTION

Iron preparation	Condition	Transferrin saturation (%)	Activity (% of injected) Liver	Spleen	Skeleton	RBC	Plasma	Washed gut	Absorption	Total uptake*
$FeSO_4$	Control ($n = 7$)	27.40 ± 8.60	5.35 ± 4.82	1.59 ± 1.68	25.40 ± 11.50	7.57 ± 5.57	6.95 ± 4.42	11.88 ± 6.27	46.90 ± 22.39	58.78 ± 21.30
	Infused ($n = 7$)	112.10 ± 10.70	24.70 ± 16.90	0.02 ± 0.02	0.32 ± 0.42	0.05 ± 0.09	0.42 ± 0.44	21.95 ± 6.57	25.52 ± 17.67	47.50 ± 23.70
Transferrin bound	Control ($n = 7$)	36.40 ± 11.00	3.80 ± 1.50	1.70 ± 1.20	26.10 ± 8.30	10.80 ± 6.10	19.00 ± 8.40	16.60 ± 7.90	61.40 ± 18.70	78.10 ± 11.30
	Infused ($n = 7$)	109.70 ± 9.80	29.20 ± 19.50	0.02 ± 0.01	0.23 ± 0.15	0.05 ± 0.06	0.33 ± 0.28	26.40 ± 8.30	29.90 ± 19.70	56.30 ± 22.70

* Activity of the washed gut segment plus absorbed activity.

of nonviable red cells in controls was 49 ± 18 and 41 ± 17%, respectively, as compared to 49 ± 10 and 40 ± 13% in hemoglobin-infused animals and 50 ± 11 and 37 ± 15% in iron-infused animals.

Hemoglobin. The ^{59}Fe hemoglobin–haptoglobin was prepared from the ^{59}Fe-labeled red cells as previously described (10). The administered dose contained 0.54 ± 0.7 mg of hemoglobin which was suspended in 0.5 to 1 ml of plasma obtained from animals treated with turpentine (0.25 ml intramuscular) 24 hr before so as to increase the haptoglobin concentration. The adequacy of haptoglobin binding was shown by a radioactive localization in the kidney of <1% when the animals were sacrificed 3 hr later. A $T_{1/2}$ plasma disappearance of the tagged hemoglobin of 15.5 ± 0.5 min was found in normal rats.

Additional experiments were conducted to compare marrow uptake of radioiron from transferrin with marrow uptake from hemoglobin in iron-infused animals. The rats were sacrificed at 3 hr and the marrow was expressed from the long bones into a phosphate-buffered saline solution at pH 7.4. Heme was extracted in ethyl acetate and recovery was shown to be more than 95% (12). Total marrow activity and heme activity were determined.

Plasma iron and iron binding capacity were performed by methods described by the International Standardization Committee (13) and by Cook *et al.* (14). Hematocrit was done by the micromethod. Results are expressed as the mean ± 1 standard deviation.

Results. *1. Iron absorption.* The effect of iron infusion on absorption of radioiron is shown in Table I. There was a similar reduction of radioiron absorption in iron-infused animals regardless of the form of iron administered. In animals receiving ferrous sulfate mean absorption was reduced from 46.9 to 25.5%, while in animals receiving diferric transferrin absorption was reduced from 61.4

TABLE II. MUCOSAL IRON CONTENT AS AFFECTED BY IRON INFUSION

Luminal iron	Condition	Nonheme iron (μg)	Iron from lumen (μg)[a]	Non-luminal iron (μg)[b]
$^{59}FeSO_4$	Controls	6.9 ± 2.5	1.5 ± 0.7	5.4 ± 2.1
	Infused	12.6 ± 6.7	2.6 ± 0.9	10.0 ± 6.8
^{59}Fe differric transferrin	Controls	8.8 ± 4.6	1.8 ± 0.7	7.0 ± 4.2
	Infused	14.2 ± 3.5	3.2 ± 1.2	11.0 ± 3.4

[a] Derived from 15 μg [test dose of iron × (mucosal activity/total activity administered)].
[b] Total mucosal iron minus iron from intestinal lumen but including iron from IV infusion.

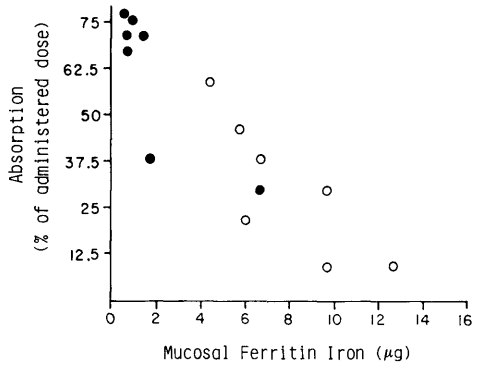

FIG. 1. Relationship between iron absorption and mucosal ferritin iron. Closed circles represent normal rats and open circles, rats infused with iron for 6 hr. There was an inverse correlation ($r = -0.895$).

to 29.9%. The absorbed iron localized in the liver and virtually no iron was found in the plasma or erythron. The iron-infused animals had increased amounts of mucosal iron (Table II). An inverse relationship was demonstrated between mucosal ferritin iron and absorption (Fig. 1). In additional studies in which infused iron was labeled with ^{55}Fe and absorbed iron with ^{59}Fe, the mucosa was divided into an outer fraction composed of cells shaken free from the villous tips and an inner fraction representing the residual scraped mucosa. Based on specific activity, it was calculated that 0.2 ± 0.2 μg of iron was derived from absorption and 0.1 ± 0.05 μg derived from infusion, or a ratio of 2. The remaining mucosa showed an iron content of 0.52 ± 0.22 μg derived from absorption and 1.8 ± 0.98 μg derived from infusion, or a ratio of 0.29.

2. Reticuloendothelial (RE) processing of nonviable red cells. The half-time disappearance of heat-damaged red cells from the plasma was 17.2 ± 4.8 min, so that at 3 hr little residual activity could be attributed to the continued presence of these cells in circulation. At this time, control animals had 37.1% of injected activity in the erythron (skeleton plus circulating red cells) and there was an additional 16.5% in the plasma (Table III). Iron-infused animals given nonviable cells had 2.2% in the erythron and 2.5% of the activity in plasma. Thus over 90% of the expected red cell uptake was blocked by the iron infusion.

This did not exclude the possibility that during red cell catabolism, either free iron or hemoglobin might be released from the RE cell, either of which would be taken up rapidly by the hepatocyte (5). The possibility of this exchange was examined by comparing splenic activity at 30 min with that at 3 hr. As shown in Table IV, ^{59}Fe activity in the spleen of iron-infused animals decreased 6.2% between 30 min and 3 hr. If it is assumed that the radioiron in residual nonviable red cells still circulating is distributed between spleen and liver as before in a 2:1 ratio, one would anticipate the 3-hr splenic activity to be some 10% greater than the 30-min value. Thus, some 16% of splenic activity appeared to have left the spleen and accumulated in the liver. This might be explained either by the release of iron from the spleen with a secondary hepatic uptake or by temporary sequestration of red cells in the spleen with subsequent intravascular hemolysis.

These possibilities could be distinguished by the infusion of hemoglobin. That resulted in an increased proportion of plasma hemoglobin being excreted by the kidney, thereby reducing the proportion taken up by the liver. In these studies, the injected hemoglobin was tagged with ^{55}Fe so that the proportion excreted could be determined. Since only 36% of the ^{55}Fe hemoglobin was excreted, less than half of the

TABLE III. EFFECT OF IRON INFUSION ON REDISTRIBUTION OF RED CELL IRON AT 3 hr

Condition	Transferrin saturation (%)	Organ distribution (% of injected activity)				
		Liver	Spleen	Skeleton	RBC	Plasma
Control ($n = 5$)	32.0 ± 20.0	19.4 ± 6.0	25.8 ± 16.0	28.0 ± 6.7	9.1 ± 2.2	16.5 ± 9.8
Iron infused ($n = 8$)	129.0 ± 23.0	46.0 ± 17.0	46.5 ± 10.2	1.6 ± 0.6	0.9 ± 0.5	2.9 ± 1.3

TABLE IV. THE EFFECT OF hb INFUSION ON NONVIABLE RED CELL IRON TIME DISTRIBUTION IN RATS RECEIVING AN INTRAVENOUS INFUSION OF IRON

Time of sacrifice	Transferrin saturation (%)	Tag	Activity (% of injected)					
			Liver	Spleen	Skeleton	RBC	Plasma	Kidney and urine
30 min (n = 7)	117 ± 22	^{59}Fe-HDRC[a]	23.7 ± 8.9	53.0 ± 12.6	1.3 ± 0.6	14.5 ± 5.9[b]	4.7 ± 3.0	2.2 ± 0.9
		^{55}Fe-Hb	3.7 ± 1.9	—	—	0.9 ± 1.0	51.4 ± 11.1	28.7 ± 9.0
3 hr (n = 7)	111 ± 16.3	^{59}Fe-HDRC[a]	35.9 ± 17.5	46.8 ± 20.2	1.7 ± 1.1	0.7 ± 0.8	4.6 ± 2.0	9.9 ± 3.3
		^{55}Fe-Hb	5.2 ± 2.4	—	—	0.4 ± 0.6	19.4 ± 3.5	35.9 ± 7.8

[a] Heat-damaged red cells.
[b] Presumably largely residual nonviable RBC.

free hemoglobin liberated from ^{59}Fe-tagged nonviable red cells would be found in the urine. The finding of 7.7% of ^{59}Fe activity in the kidney and urine of hemoglobin-injected seem inconsistent with previous observations animals during the interval between 30 min and 3 hr (Table IV) meant that hemolysis could account for some 15% of red cell breakdown, precisely the increase in hepatic activity.

3. Hepatocyte processing of hemoglobin iron. The injected hemoglobin–haptoglobin iron had a clearance $T_{1/2}$ of 15.5 ± 0.5 min from plasma. When combined with haptoglobin, hemoglobin is known to be selectively removed by the hepatocyte (5). In control animals at 3 hr, 66.3% was in the liver, 27.2% in the erythron, and 4.9% in plasma. In iron-infused animals, 80.9% was in liver, 13.2% in the marrow, and 1.5% in plasma (Table V). The very low activity in plasma and absence of activity in circulating red cells suggested a complete block in iron supply from hepatocyte to erythron, yet significant activity was present in the marrow. To evaluate this, the bone marrow was examined for heme and nonheme iron in iron-infused animals given tagged hemoglobin and in animals receiving iron-tagged transferrin. A much reduced utilization of hemoglobin iron for hemoglobin synthesis by the erythroid cells of the marrow was found in the hemoglobin and the iron-injected animals (Table VI).

Discussion. Iron is supplied to plasma transferrin by the intestinal mucosa, by RE cells, and by hepatocytes. In this study, radioiron was introduced into these three types of cells and its release was examined in rats in the presence of unsaturated and saturated transferrin.

The behavior of the intestinal mucosa was evaluated by injecting ferrous sulfate or transferrin-bound iron into the duodenum with determination of mucosal uptake and distribution into body tissues. While most iron may be absorbed as the iron salt, there is evidence that luminal transferrin may mediate at least part of iron absorption (8). It has the advantage of less adsorption to the mucosal surface and eliminates the possibility of absorption by diffusion. There have been studies indicating that the degree of plasma transferrin saturation has little or no effect on the amount of iron ab-

TABLE V. EFFECT OF IRON INFUSION ON TISSUE DISTRIBUTION OF HEMOGLOBIN–HAPTOGLOBIN AT 3 hr

Condition	Transferrin saturation (%)	Organ distribution (% of injected activity)				
		Liver	Spleen	Skeleton	RBC	Plasma
Control (n = 5)	39.0 ± 6.0	64.3 ± 4.0	3.0 ± 0.8	23.3 ± 4.1	3.9 ± 1.9	4.9 ± 1.8
Iron infused (n = 7)	134.0 ± 36.0	80.9 ± 6.3	3.8 ± 1.0	13.2 ± 1.3	0	1.6 ± 0.5

sorbed, but that complete saturation of transferrin affects the internal distribution by diverting absorbed iron to the hepatocyte (15–18). In the present study, iron was absorbed despite transferrin saturation, but the amount was reduced by about 50%. This would not since the prolonged period of iron infusion resulted in an increase in mucosal iron and ferritin content. While there are other reports of similar blocking due to mucosal iron loading from parenteral or oral doses of iron (19, 20), the present study demonstrates the rapidity with which this block may be produced. Crosby and associates presented evidence that the iron content of mucosal cells might regulate absorption (21), but subsequent studies produced variable results (22–24). However, added support is provided by the relation between absorption and mucosal ferritin iron in these studies and by measurements of mucosal ferritin from intestinal biopsies in man (25).

Reticuloendothelial processing of iron can be best examined in the spleen. At least half of the nonviable red cells are localized to that organ, while the spleen would be expected to take up little transferrin iron, nonspecifically bound iron, or hemoglobin. Iron infusion impaired the release of red cell activity from the spleen, the amount going to the erythron being only 7% that of the control (Table III). Over the 3-hr period after injection of nonviable cells, some decrease in splenic activity was observed, whereas some increase due to processing of remaining circulating nonviable red cells might be expected. Additional studies in which infused hemoglobin was used to divert plasma hemoglobin into the urine suggested that the decreased splenic activity and reciprocal increase in liver radioiron in the prior studies was due to intravascular hemolysis of red cells temporarily held in the splenic pulp. Thus it may be concluded that the RE cell requires unsaturated transferrin to unload iron once it is processed from hemoglobin. This is consistent with previous observations showing the desferrioxamine was unable to interfere with the movement of iron from RE to transferrin to erythroid marrow (11).

TABLE VI. THE NATURE OF MARROW RADIOIRON AFTER ^{59}Fe-TAGGED HEMOGLOBIN–HAPTOGLOBIN OR TRANSFERRIN INJECTION

Tag	Condition	Skeletal activity (% of injected dose)	
		Heme	Nonheme
Intravenous ^{59}Fe hemoglobin–haptoglobin	Control (n = 6)	13.0 ± 3.7	10.5 ± 2.2
	Iron infused (n = 6)	1.6 ± 1.1	11.9 ± 2.6
Intravenous transferrin	Control (n = 4)	36.6 ± 7.7	13.8 ± 4.0
	Iron infused (n = 4)	21.3 ± 8.4	19.7 ± 1.6

On the basis of these studies, it seems appropriate to postulate a donor site on the membrane of the RE cell with which unsaturated transferrin reacts to obtain iron. This can explain certain aspects of iron store distribution. In patients with aplastic anemia, transfused red cell iron initially localizes largely in the RE cells (26). While red cell destruction in such patients is either normal or somewhat increased, ferrokinetic studies indicate that transferrin iron turnover is reduced to about one-third of that being processed (M. Cazzola and C. A. Finch, unpublished data). Thus nearly two-thirds of iron processed by RE cells will accumulate in that cell. The de-

pendency for RE release on unsaturated transferrin also explains changes in iron distribution in idiopathic hemochromatosis. In the early stages of this disease, RE iron stores are not enlarged, presumably due to an enhanced release of iron from that cell (27). However, when transferrin is saturated with iron late in the disease, RE iron accumulates, presumably due to the limitation in plasma iron transport capacity.

It is known that hemoglobin–haptoglobin in the plasma is for the most part cleared by the hepatocyte (5, 10, 11). The higher liver localization and reduced skeletal activity indicated that rerouting of this iron to the erythroid marrow was prevented when transferrin was saturated. The sequestration of hemoglobin iron in the liver of the transferrin saturated animal has an analogy to patients with ineffective erythropoiesis who have hemoglobinemia and developed hepatocyte overload (28).

The absence of detectable red cell activity in iron-infused animals injected with radioactive hemoglobin suggests that the block should be complete, but some 13% was found in the skeleton. To evaluate the possibility that some of the injected rat hemoglobin may have polymerized and been phagocytosed by reticuloendothelial cells, additional studies were carried out to determine whether iron taken up by the marrow was utilized for hemoglobin synthesis or stored as ferritin. The disproportionate increase of ferritin iron in hemoglobin–haptoglobin-infused animals as compared to animals given transferrin iron (Table VI) provided support for the above suggestion.

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