

## Sequence of development of iron deficiency in the rat<sup>1, 2</sup>

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**ABSTRACT** This study was designed to determine the interrelationships among storage iron, transport iron, and iron compounds that serve known physiological functions (Hb, myoglobin, and cytochrome c) during the gradual progression of dietary iron deficiency. These three categories of iron compounds are generally considered to become depleted in three corresponding, sequential stages. However, there is scattered evidence of substantial overlap between these stages in man. The presence of such overlap may prove pertinent to the interpretation of laboratory tests used in the diagnosis of iron deficiency. The rat was used as an experimental model to allow more complete evaluation of the interrelationships between the stages of iron deficiency than would be possible in man. Rats were given diets containing 2, 6, and 50 mg iron/kg diet during early adult development, between 36 and 90 days of age. The iron-deficient diets (2 and 6 mg iron/kg diet) resulted in decreases in hematocrit and in muscle and intestinal cytochrome c well before storage iron in the liver and spleen was exhausted. The results in the rat model may help to explain why there is not a consistent pattern of laboratory abnormalities in individuals with chronic, mild dietary iron deficiency. *Am J Clin Nutr* 1982;35:671-677.

**KEY WORDS** Iron deficiency, hematocrit, myoglobin, cytochrome c; sequence and stages

### Introduction

Dietary iron deficiency is generally considered to progress in a sequence of three stages (1, 2). The first stage consists of depletion of iron stores, the next stage is characterized by a decrease in transport iron, and the last occurs with diminished production of iron proteins that serve known physiological functions (Hb, myoglobin, and iron-containing enzymes). The laboratory tests that are used to diagnose iron deficiency can also be classified according to which of the three stages they identify. A decline in the concentration of serum ferritin accompanies the loss of storage iron. The fall in serum iron and rise in iron binding capacity results in a decline in the iron saturation of transferrin which signifies impaired iron transport. And, lastly, anemia, a rise in erythrocyte protoporphyrin and a decrease in red cell volume are indicative of the final stage of iron deficiency, the restricted production of Hb and other iron compounds that fulfill recognized physiological functions. Although such a classification provides a convenient conceptual framework, the laboratory tests do not appear to conform strictly to this pattern in individual patients

with mild iron deficiency. For example, the concentration of serum ferritin, since it reflects iron stores, might be expected to be abnormal in all patients with iron-responsive anemia, unless they had some complicating illness. Yet in infants with mild iron-responsive anemia, serum ferritin values were often found to be in the normal range (3, 4). In both infants and adults there was evidence of marked overlap between iron-deficient and normal populations not only in serum ferritin values, but also in red cell volume, erythrocyte protoporphyrin, and transferrin saturation (4, 5). Another line of evidence for overlap between the stages of iron deficiency comes from the relationship of serum iron, iron-binding capacity, and transferrin saturation to storage iron. Serum iron and transferrin saturation are lower in children (6, 7) and possibly in women (8, 9) who are not

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iron deficient but who normally have lower iron stores than men. Furthermore, iron-binding capacity appears to be increased in individuals with diminished but not exhausted iron stores (10). All of these findings suggest that there is considerable overlap between the stages of iron deficiency and/or that biologic variability and analytic errors for laboratory tests substantially impair the reliability with which they reflect their corresponding stages of iron deficiency in individual patients.

Studies of the progression of iron deficiency have been done primarily in adult males who were subjected to repeated phlebotomy (11-13). The reason for this approach is that dietary iron deficiency in adults progresses too slowly to lend itself readily to experimental observation. The progression is more rapid in infants (14); nevertheless, obtaining samples of solid tissues is rarely justifiable in man. We therefore undertook the present study in the rat model to provide more information on the sequence of development of dietary iron deficiency. We recognize that there are major quantitative differences between the rat model and man; the rat, for example, because of its rapid growth rate, derives a greater percentage of iron from the diet than from the recycling of Hb iron. Nevertheless, the many qualitative similarities of iron metabolism in the rat have justified its widespread use as an experimental model.

## Materials and methods

### Rats

Male Sprague-Dawley rats were obtained from Simonsen Laboratories, Gilroy, CA on the day of weaning at 21 days of age. They were housed in rust-free cages under a 12-hr light-dark cycle. Food and distilled water were given ad libitum.

### Diets

A semipurified diet (Teklad, Madison, WI) was used in all experiments. It was identical in composition to that recently proposed for growing rats by a committee of the American Institute of Nutrition (15) except that cellulose was omitted since it is an ingredient of variable iron content. Rats were given diets containing 2, 6, 50, or 100 mg of iron/kg of diet; only the iron content was varied and iron was added as ferric citrate. Our analyses verified that the diet without addition of iron contained 2 mg of iron/kg of diet and that iron content of the diets corresponded to those requested from the manufacturers.

### Blood sampling

All blood samples were obtained from the tail. In order to facilitate free flow of blood from the tail, rats were maintained at an environmental temperature of 37°C for a 10-min period before sampling.

### Tissue sampling

Whole livers were removed, weighed, and then frozen at -18°C. Muscle samples for cytochrome c were taken from both thighs, after careful separation from bones and fat; muscles for analysis of myoglobin were from the abdominal wall. Intestinal mucosa was obtained by scraping the upper half of the small intestine with a spatula after gentle removal of the bowel contents. Muscle and intestinal mucosa were frozen for a maximum of 3 months before analysis. This period of storage did not result in a significant loss of myoglobin or cytochrome c.

### Analyses

Hematocrit was determined by centrifugation of blood in heparinized glass capillary tubes. Serum iron and total iron-binding capacity were measured in serum that was separated from the cells within 2 h of blood drawing and then stored frozen. Analyses were performed using Ferrozone as the color reagent and using the Technicon Autoanalyser II (16). For the spectrophotometric analysis of myoglobin, Hb was separated from myoglobin by chromatography on diethylaminoethyl cellulose columns (17). Concentration of cytochrome c was measured spectrophotometrically after sonication of muscle and intestine homogenates (18). Liver nonheme iron was determined after acid hydrolysis (19). Ferritin iron was estimated after heat treatment of tissue homogenates as in earlier studies (20), but ammonium sulfate precipitation was added as an additional purification step (21).

### Statistical analysis

Groups of six to eight rats were used in all experiments. The significance of differences between groups was estimated by Student's *t* test. SEM are provided in the text and tables.

### Experimental conditions

Starting at 21 days of age rats were given a diet containing 100 mg iron/kg diet. This is four times the iron level that was previously found to be required for maximal concentrations of Hb and skeletal muscle cytochrome c and myoglobin under the same conditions (20). This high level of dietary iron was intended to establish ample iron stores before the initiation of iron deficient diets at 36 days of age. At 36 days of age, the rats were randomly divided into three groups, one of which was given a control diet containing 50 mg iron/kg and two others which received either 2 or 6 mg iron/kg diet. Six to eight rats from each of the diet groups were killed by decapitation at 36, 39, 42, 50, 60, 75, and 90 days of age (0, 3, 6, 14, 14, 39, and 54 days after initiation of the three iron regimens). The iron content of the diets did not substantially influence body weight; this allowed us to study those blood and tissue effects of iron deficiency that are independent of the secondary effects that severe iron deficiency has on growth when the dietary regimen

is started at an earlier age (22) when rats were randomized to diets at 36 days of age, the mean body weight in the 50 mg/kg group compared with the 2 mg/kg group, was there any weight differences that approached border (0.05).

## Results and discussion

### Storage iron

The concentration of heme iron in the liver of rats (µg iron/kg diet) remained between mean values of 62 and 75 µg/kg during the 54-day study period. This was substantial and significant at 6 days with both of the iron deficient regimens (Table 1 and Figure 1). This decline is partly attributable to the rate of growth. The concentration of nonheme iron at 6 days was 50 µg/kg group than in the control group. Thereafter, values in the two iron deficient groups decreased to very low values that were maintained throughout the 54 days of the dietary regimen.

In the spleen, the development of nonheme iron followed a similar pattern from the liver values in that the concentration of about 40 µg/g in the control group at the early stages of the study increased during the study period (Table 1). The developmental patterns in the liver and spleen in accord with the concentration of iron exists in two major com-

TABLE 1  
Nonheme iron, (µg iron/g tissue)

Days of regimen	Dietary iron (mg/kg)	
	2	50
	days	
0	36	69 ± 10
3	39	85 ± 11
6	42	85 ± 16
10	46	76 ± 3
14	50	64 ± 8
24	60	62 ± 8
39	75	80 ± 11
54	90	68 ± 8

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of the small intestine with a  
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were frozen for a maximum of  
This period of storage did not  
of myoglobin or cytochrome c.

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the cells within 2 h of blood  
frozen. Analyses were per-  
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is started at an earlier age (22). In the present study, when rats were randomized to the three dietary groups at 36 days of age, the mean body weight was  $138 \pm 5$  g. Only at 90 days of age, when mean weight was  $373 \pm 10$  g in the 50 mg/kg group compared to  $351 \pm 10$  in the 2 mg/kg group, was there any weight difference between groups that approached borderline significance ( $p \sim 0.05$ ).

## Results and discussion

### Storage iron

The concentration of hydrolyzable nonheme iron in the liver of control rats (50 mg iron/kg diet) remained almost constant, between mean values of 62 and 85  $\mu\text{g/g}$  during the 54-day study period. In contrast, there was substantial and significant decline after 6 days with both of the iron-deficient regimens (Table 1 and Figure 1). The rapidity of this decline is partly attributable to a rapid rate of growth. The concentration of liver nonheme iron at 6 days was lower in the 2 mg/kg group than in the 6 mg/kg group; thereafter, values in the two groups continued to decrease to very low and almost identical values that were maintained between 14 and 54 days of the dietary regimens.

In the spleen, the developmental changes in nonheme iron followed a different pattern from the liver values in the control rats. Low values of about 40  $\mu\text{g/g}$  were present in the control group at the earlier time points, but the concentration increased over 10-fold during the study period (Table 1). The disparate developmental patterns of nonheme iron in liver and spleen in the control group are in accord with the concept that storage iron exists in two major compartments (23). The

larger of the two compartments is in the hepatocyte and the smaller is in the reticulo-endothelial (RE) system. The spleen RE cells are involved in removal of senescent red cells from the blood. The rise in concentration of nonheme iron in the spleen can probably be explained by the fact that relatively few red cells will reach senescence until after 60 days of age [the estimated life span of the red cells in the rat (24) is about 60 days] and only then can one expect RE iron to be substantially augmented. Before that time, the breakdown of the relatively small number of red cells that were synthesized in fetal life cannot make a substantial contribution to the iron stores of an animal that has had an over 50-fold increase in body weight.

Spleen nonheme iron fell significantly below control values after 3 days in the 2 mg iron/kg group and after 6 days in the 6 mg iron/kg group. After this, the value in both deficient groups remained similarly low and relatively constant, but the difference compared to the control group continued to increase by virtue of the marked rise in values of the control animals.

Nonheme iron in intestinal mucosa declined in the control group from initial concentrations of 49  $\mu\text{g/g}$  to final values of 10  $\mu\text{g/g}$ . In both deficient groups, values fell markedly and significantly within 3 days, reached similar very low values after 6 days, and subsequently fell below the sensitivity of the assay. This very prompt change is made possible by the rapid turnover of the mucosal cells; the capacity for renewing this population of cells within 2 days (25) allows this tissue to reflect changes in iron status very

TABLE 1  
Nonheme iron, ( $\mu\text{g}$  iron/g tissue)

Dietary iron (mg/kg)	Liver			Spleen			Intestinal Mucosa			
	50	6	2	50	6	2	50	6	2	
Days of regimen	Age									
	days									
0	36	69 $\pm$ 10			40 $\pm$ 3			49 $\pm$ 2		
3	39	85 $\pm$ 11	74 $\pm$ 12	71 $\pm$ 10	56 $\pm$ 4	49 $\pm$ 4	38 $\pm$ 4	49 $\pm$ 4	14 $\pm$ 1	23 $\pm$ 6
6	42	85 $\pm$ 16	35 $\pm$ 6	22 $\pm$ 3	40 $\pm$ 6	23 $\pm$ 4	17 $\pm$ 2	27 $\pm$ 6	8 $\pm$ 1	8 $\pm$ 1
10	46	76 $\pm$ 3	22 $\pm$ 4	17 $\pm$ 3	57 $\pm$ 11	20 $\pm$ 6	15 $\pm$ 1	30 $\pm$ 7	2 $\pm$ 1	3 $\pm$ 1
14	50	64 $\pm$ 8	14 $\pm$ 3	12 $\pm$ 1	55 $\pm$ 5	14 $\pm$ 1	12 $\pm$ 1	31 $\pm$ 4	<1	<1
24	60	62 $\pm$ 8	7 $\pm$ 1	8 $\pm$ 2	93 $\pm$ 14	14 $\pm$ 1	15 $\pm$ 2	22 $\pm$ 5	<1	<1
39	75	80 $\pm$ 11	8 $\pm$ 2	10 $\pm$ 1	228 $\pm$ 22	21 $\pm$ 1	18 $\pm$ 2			
54	90	68 $\pm$ 8	13 $\pm$ 1	13 $\pm$ 1	426 $\pm$ 49	32 $\pm$ 3	27 $\pm$ 2	10 $\pm$ 1	<1	3 $\pm$ 1

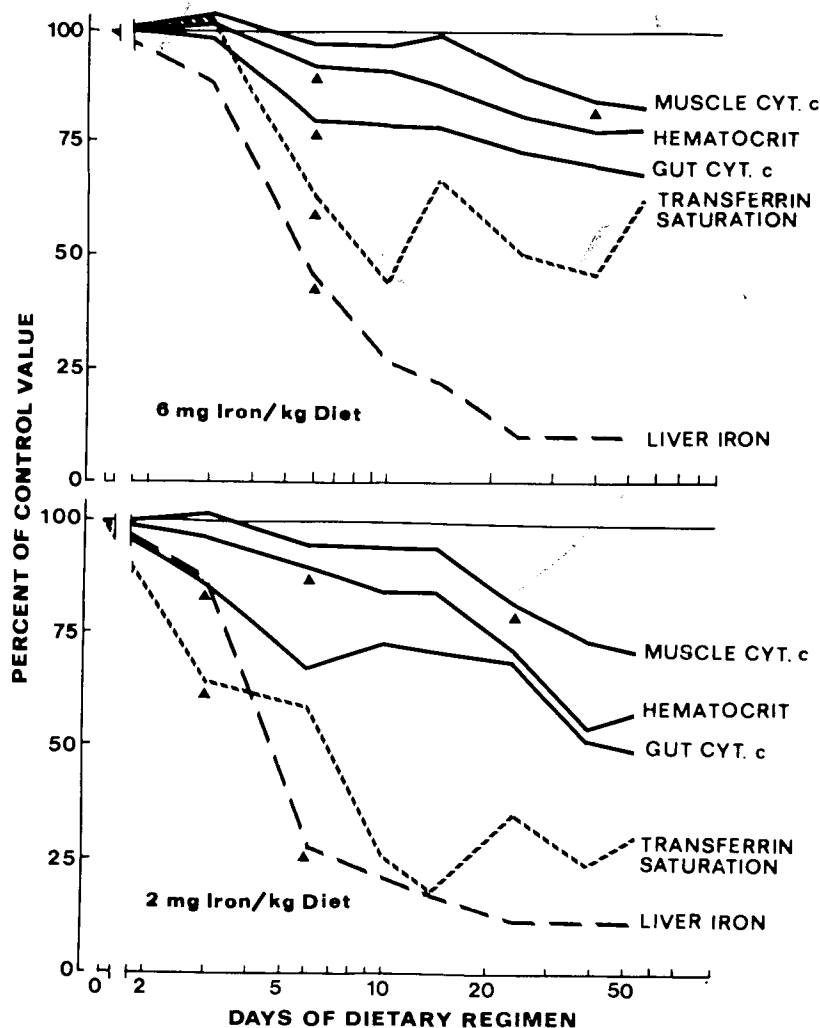


FIG. 1. Sequence of development of iron deficiency at two levels of dietary iron. The black triangles indicate the day on which differences between the iron-deficient and control groups first became significant ( $p < 0.05$ ); in all cases, the differences remained significant at all subsequent sampling days.

quickly. In addition, since the intestinal mucosa derives its iron both from systemic and intraluminal sources, the sudden decrease in the luminal iron supply would be expected to affect the mucosal iron content within a short time.

#### Transport iron

A significant decline in serum iron compared to the control group was first noted at 3 days in the 2 mg iron/kg group and at 6 days in the 6 mg/kg group; the decline continued until a plateau was reached after 14 days in the former and 24 days in the latter

(Table 2). Transferrin saturation followed a similar pattern; the changes became significant at 3 days in the 2 mg/kg group and at 6 days in the 6 mg/kg group (Fig. 1).

#### Hematocrit, cytochrome c, and myoglobin

In the control group, the hematocrit rose gradually from  $35.7 \pm 0.3$  at the 0-day point (36 days of age) to  $42.4 \pm 0.7\%$  at the 24-day point (60 days of age). Thereafter, there was only a minimal increase to  $43.6 \pm 0.4\%$  at the 54-day point (90 days of age). This represents a continuation of the developmental increase in values that is more rapid between 21 and

TABLE 2  
Transport iron

Dietary iron (mg/kg)		Age days	50
Days of regimen	Age		
0	36	329 ± 28	
3	39	464 ± 43	
6	42	298 ± 40	
10	46	394 ± 13	
14	50	320 ± 30	
24	60	239 ± 35	
39	75	225 ± 13	
54	90	209 ± 18	

35 days of age (20). In the 6 mg values remained almost constant; in the 2 mg/kg group they declined; from the control group became significant at 6 days in both groups (Table 2).

Cytochrome c in skeletal muscle was little in the control group (Table 2). In two iron-deficient groups, there was a gradual decline in muscle cytochrome c; the decrease was proportionally less marked than the increase in hematocrit (Fig. 1) and did not reach significance until 24 days of regimen in the 2 mg/kg group and 39 days in the 6 mg/kg group. The degree of cytochrome c depletion, as the anemia, was more study than in otherwise similar experiments (20), probably because of the present conditions, there were no iron stores and the rats' greater muscle associated with a slower rate of growth. The changes in skeletal muscle myoglobin followed the same pattern as for cytochrome c (Table 3). The concentration of myoglobin in both deficient groups became significantly decreased below control values after 24 days of the regimen; subsequently the depression continued to be significant ( $p < 0.001$ ) only in the 2 mg/kg group.

Cytochrome c in intestinal mucosa was deficient rapidly. In contrast to the stable values in the control group, concentrations in the 2 and 6 mg/kg groups became significantly at 3 and 6 days, respectively (Fig. 1). The percentage decline in both group values was more profound than anemia, particularly during the latter part of the study period. The disproportionate effect on intestinal cytochrome

TABLE 2  
Transport iron

Days of regimen	Age	Dietary iron (mg/kg)			Serum iron ( $\mu\text{g}/\text{dl}$ )			Transferrin saturation (%)		
		50	6	2	50	6	2	50	6	2
	days									
0	36	329 $\pm$ 28					54 $\pm$ 5			
3	39	464 $\pm$ 43	471 $\pm$ 81	278 $\pm$ 48	68 $\pm$ 5	69 $\pm$ 10	44 $\pm$ 6			
6	42	298 $\pm$ 40	195 $\pm$ 15	187 $\pm$ 35	45 $\pm$ 7	28 $\pm$ 3	27 $\pm$ 5			
10	46	394 $\pm$ 13	189 $\pm$ 37	109 $\pm$ 18	15 $\pm$ 8	29 $\pm$ 5	17 $\pm$ 3			
14	50	320 $\pm$ 30	232 $\pm$ 49	69 $\pm$ 7	51 $\pm$ 5	34 $\pm$ 7	9 $\pm$ 1			
24	60	239 $\pm$ 35	100 $\pm$ 19	65 $\pm$ 17	40 $\pm$ 5	20 $\pm$ 3	14 $\pm$ 3			
39	75	225 $\pm$ 13	78 $\pm$ 9	51 $\pm$ 11	37 $\pm$ 1	17 $\pm$ 2	9 $\pm$ 1			
54	90	209 $\pm$ 18	109 $\pm$ 17	44 $\pm$ 5	35 $\pm$ 2	21 $\pm$ 2	10 $\pm$ 1			

35 days of age (20). In the 6 mg/kg group, values remained almost constant and in the 2 mg/kg group they declined; differences from the control group became significant at the 6-day point in both groups (Fig. 1).

Cytochrome c in skeletal muscle changed little in the control group (Table 3). In the two iron-deficient groups, there was a very gradual decline in muscle cytochrome c that was proportionally less marked than the decrease in hematocrit (Fig. 1) and that did not reach significance until 24 days of the regimen in the 2 mg/kg group and 39 days in the 6 mg/kg group. The degree of cytochrome c depletion, as the anemia, was milder in this study than in otherwise similar previous experiments (20), probably because under the present conditions, there were larger initial iron stores and the rats' greater age was associated with a slower rate of growth. The changes in skeletal muscle myoglobin followed the same pattern as for cytochrome c (Table 3). The concentration of myoglobin in both deficient groups became significantly decreased below control values ( $p < 0.05$ ) after 24 days of the regimen; subsequently, the depression continued to be significant ( $p < 0.001$ ) only in the 2 mg/kg group.

Cytochrome c in intestinal mucosa became deficient rapidly. In contrast to the relatively stable values in the control group, the concentrations in the 2 and 6 mg/kg groups fell significantly at 3 and 6 days, respectively (Fig. 1). The percentage decline below control group values was more profound than the anemia, particularly during the first half of the study period. The disproportionately profound effect on intestinal cytochrome c early

in the progression of iron deficiency is probably related to the rapid cell turnover of mucosal cell which allows it to reflect sudden dietary iron changes more rapidly than the red blood cell. This explanation is in accord with the earlier finding that mucosal cytochrome c depletion in the iron-deficient rat was corrected within 2 days of iron treatment, compared to a week or more required for reversal of anemia and skeletal muscle cytochrome c and myoglobin depletion (22).

#### Overlapping stages

During the progression of iron deficiency in the 2 mg iron/kg diet group, the most marked initial change was the decline in transferrin saturation within 3 days. The concentration of cytochrome c in intestinal mucosa was also significantly depressed within 3 days. Hematocrit did not become significantly depressed until 6 days after initiation of the iron-deficient diet. The more rapid rate of turnover of the mucosal cell (2 days) compared to the red blood cell (60 days) is a plausible explanation for this difference in timing (24, 25). Although liver and spleen storage iron had decreased considerably by the 6-day point, they did not reach their minimum values until the 24-day point. Consequently, anemia and cytochrome c depletion could occur well before exhaustion of potentially mobilizable iron stores. Thus, in the rat model, there was a marked overlap between depletion of storage iron, transport iron, and iron compounds that fulfill known physiological functions.

Although the *longitudinal progression* of the three stages of iron deficiency showed

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black triangles indicate the  
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Thereafter, there was  
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developmental increase  
rapid between 21 and

TABLE 3  
Cytochrome c and myoglobin

Dietary iron (mg/kg)	Cytochrome c, $\mu\text{g/g}$						Myoglobin, ng/g							
	Muscle			Intestinal			Muscle			Muscle				
	50	2	50	6	50	2	50	6	50	2	50	6	2	
Days of regimen	50	2	50	6	50	2	50	6	50	2	50	6	2	
Age	50	6	50	6	50	2	50	6	50	2	50	6	2	
days	0	36	39	42	46	50	24	39	54	36	39	42	46	50
	125 $\pm$ 3.2	121 $\pm$ 2.3	130 $\pm$ 4.6	128 $\pm$ 3.0	122 $\pm$ 2.9	133 $\pm$ 5.6	130 $\pm$ 4.1	132 $\pm$ 3.6	129 $\pm$ 2.1	125 $\pm$ 3.9	125 $\pm$ 3.9	123 $\pm$ 3.1	122 $\pm$ 3.6	118 $\pm$ 4.3
	109 $\pm$ 5.8	109 $\pm$ 5.8	109 $\pm$ 5.8	109 $\pm$ 5.8	109 $\pm$ 5.8	109 $\pm$ 5.8	109 $\pm$ 5.8	109 $\pm$ 5.8	122 $\pm$ 3.9	124 $\pm$ 2.6	124 $\pm$ 2.6	122 $\pm$ 3.3	116 $\pm$ 3.4	109 $\pm$ 3.9
	99 $\pm$ 2.6	117 $\pm$ 3.0	118 $\pm$ 7.5	111 $\pm$ 3.4	105 $\pm$ 4.6	85 $\pm$ 7.0	85 $\pm$ 5.1	105 $\pm$ 5.8	111 $\pm$ 3.4	111 $\pm$ 3.4	111 $\pm$ 3.4	111 $\pm$ 3.4	111 $\pm$ 3.4	105 $\pm$ 4.6
	113 $\pm$ 4.9	93 $\pm$ 2.5	87 $\pm$ 1.0	82 $\pm$ 4.0	62 $\pm$ 4.3	59 $\pm$ 4.6	71 $\pm$ 2.6	113 $\pm$ 4.9	100 $\pm$ 2.7	80 $\pm$ 1.7	81 $\pm$ 3.4	75 $\pm$ 2.3	59 $\pm$ 2.0	45 $\pm$ 2.0
	1.5 $\pm$ 0.10	1.5 $\pm$ 0.06	1.5 $\pm$ 0.04	1.8 $\pm$ 0.07	1.5 $\pm$ 0.10	1.6 $\pm$ 0.06	1.7 $\pm$ 0.12	1.9 $\pm$ 0.08	1.5 $\pm$ 0.08	1.4 $\pm$ 0.12	1.7 $\pm$ 0.05	1.4 $\pm$ 0.13	1.4 $\pm$ 0.13	1.3 $\pm$ 0.09
	1.5 $\pm$ 0.08	1.4 $\pm$ 0.12	1.4 $\pm$ 0.07	1.6 $\pm$ 0.08	1.6 $\pm$ 0.08	1.5 $\pm$ 0.05	1.6 $\pm$ 0.08	1.5 $\pm$ 0.05	1.5 $\pm$ 0.08	1.4 $\pm$ 0.12	1.4 $\pm$ 0.07	1.4 $\pm$ 0.07	1.4 $\pm$ 0.07	1.3 $\pm$ 0.07

marked overlap, a *cross-sectional comparison* of the two iron-deficient groups at any time point after 14 days would give the impression that depletion of storage iron represented an initial stage of iron deficiency. During this period between 14 and 54 days storage iron was equally depressed regardless of the severity of the iron deficiency. The differences in severity were expressed in transferrin saturation, hematocrit, cytochrome c, and myoglobin. Animals studied at any one of these later time points would seem to have the same degree of depletion of iron stores as a prerequisite for the other changes of iron deficiency, whether the deficiency was severe or mild. This latter portion of the dietary regimens comes closer to the type of equilibrium state in which dietary iron deficiency is seen in man, where the condition has progressed very gradually rather than as a result of a sudden change in iron balance.  $\square$

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