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Iron supplementation moderates but does not cure the Belgrade anemia

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Belgrade rats inherit microcytic, hypochromic anemia as an autosomal recessive trait (gene symbol *b*). Erythrocytes and tissue are iron deficient in the face of elevated TIBC (total iron binding capacity) and percent iron saturation; iron injections increased the number of erythrocytes but their appearance remained abnormal. We have investigated iron supplements to improve husbandry of *b/b* rats and to learn more about the underlying defect and its tissue distribution. Weekly IM (intramuscular) injections of iron–dextran (Imferon at 30 mg kg^{-1}) improved the anemia but did not alter the red cell morphology. Certain diets also improved the health of *b/b* rats when compared to standard rat chows by the criteria of weight, survival to adulthood, hematology and reproduction. The critical nutritional factor turned out to be iron bioavailability, with ferrous iron added to the diet improving the health of Belgrade rats without affecting the underlying erythroid defect. Tissue iron measurements after dietary or parenteral supplementation confirmed the iron deficient status of untreated *b/b* rats and established that dietary ferrous iron partially relieved this deficiency, with injections leading to greater amounts of tissue iron. Serum iron and TIBC were also found to be elevated in untreated *b/b* rats, with dietary supplementation decreasing but not eliminating the elevation in TIBC. These studies indicate that iron supplements can improve the health of *b/b* rats without altering the underlying defect and also suggest that the mutation could alter iron uptake in the GI (gastrointestinal) tract.

Keywords: Belgrade rat, dietary iron, ferrous, parenteral iron, serum iron

Introduction

The Belgrade rat has a radiation induced mutation (Pavic *et al*. 1961, Sladic-Simic *et al*. 1963) that manifests as microcytic, hypochromic anemia inherited as an autosomal recessive trait (Sladic-Simic *et al*. 1966, 1969). These reports described apparent iron deficiency in erythrocytes and other tissues despite elevated TIBC (total iron binding capacity) and iron saturation. Parenteral iron increased the RBC (red blood cell) count but cell morphology remained abnormal. Because we had observed that certain diets (below) also elevated RBCs with little improvement in microcytosis or hypochromia, we have reinvestigated iron supplementation to: (1) improve the husbandry of this fragile, but interesting mutant; (2) help understand the underlying defect; and (3) learn whether iron uptake is impaired only in erythroid cells or whether the GI (gastrointestinal) tract also has defective iron uptake. Examination of growth, hematological parameters, tissue iron, serum iron and TIBC has revealed that iron supplementation improves the husbandry of *b/b* rats without altering the underlying defect. The data also permit inferences about the defect, including the fact that it may affect dietary iron utilization in addition to its well documented effects on erythroid

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iron metabolism (Edwards *et al*. 1978, Bowen & Morgan 1987, Garrick *et al*. 1991, 1993a, b, Farcich & Morgan 1992a, b). Brief preliminary accounts of this research have been presented earlier (Garrick *et al*. 1994, 1995).

Materials and methods

Rat colony

Belgrade rats inherit a hypochromic, microcytic anemia as an autosomal recessive trait; thus homozygotes (*b/b*) can be compared to phenotypically normal heterozygotes (+/*b*). Most comparisons in this study are between littermates. Each experimental group contained both male and female rats; although this choice increased the variance for certain measurements such as body weight, it was deemed necessary to provide sufficient numbers given the small litter sizes. Iron–dextran was administered IM (intramuscularly) weekly as Imferon (Fisons, Loughborough, UK) at 30 mg kg^{-1} where indicated. Blood was collected after gentle anesthesia by retro-orbital sinus puncture or superficial venesection (for pre-weanling pups). Normal diet consisted of Purina rat chow (Ralston-Purina, St. Louis, MO) or Agway CRM 2000 (Syracuse, NY). Breeder diets were Teklad Mouse Breeder Chow (Madison, WI) or Agway CRM 3000 or 3200. Teklad TD77450, a control diet for a corresponding iron deficient one, was also used. Supplemental iron was added as reagent grade chemicals (such as $FeSO_4$, listed for the individual experiments) except for iron carbonate which was purchased as Feed Grade from Harlan Teklad. Diets were prepared by adding the iron compounds to the powdered diet and mixing initially in 500 g lots in a Waring Blender, later in 9 kg lots in a Hobart Mixer. Other food supplements were purchased from US Biochemicals (Cleveland, OH). Water was supplied *ad lib*. Animal experiments were reviewed by the SUNY at Buffalo IACUC.

Hematological measurements

Heparinized blood was characterized as follows: PCV (packed cell volume) was measured after centrifugation in a micro-hematocrit centrifuge; Hb (hemoglobin) was determined spectrophotometrically after dilution into Drabkin's reagent; RBCs were diluted in saline and counted on a Coulter Model Z_F counter with appropriate settings for rat erythrocytes; reticulocytes were stained with Brilliant Cresyl Blue, then at least 1000 erythrocytes were counted per determination under an oil immersion objective; MCV (mean cellular volume), MCH (mean cellular hemoglobin) and MCHC (mean cellular hemoglobin concentration) were calculated from PCV, Hb and RBC.

Iron determinations

Tissue nonheme iron was determined as previously described (Garrick *et al*. 1989); care was taken when working with liver to combine several slices well distributed across the tissue to get a representative iron density for each rat. Serum iron, % iron saturation and TIBC were determined spectrophotometrically as ferrozine complexes using a kit purchased from Sigma (St. Louis, MO) and adapting the procedure for use with the smaller volumes of serum obtained from rats.

Statistical analyses

Statistical analyses relied on Stata (Stata Corp., College Station, TX). Choice of method is indicated with the result or its discussion. $P \leqslant 0.05$ was treated as significant.

Results

Parenteral iron

Sladic-Simic *et al*. (1966) initially reported that parenteral iron increased the RBC count of *b/b* rats but had little effect on cell morphology. This observation could mean that GI iron uptake was inadequate, but seemed contradictory to their observation of elevated percent iron saturation (Sladic-Simic *et al*. 1969). Bannerman and Hoke (personal communication via Hoke) had failed to see such an improvement after iron injection so we reinvestigated parenteral iron supplementation. Three month old rats were bled on day 0 (just before the first iron injection) and at approximately weekly intervals thereafter. The hematological data and growth responses of the animals are shown in Figure 1. Normal (+/*b*) rats exhibit normal values for PCV (A), Hb (B), RBC (C), MCH (D), MCV (E), MCHC (F), weight (G) and reticulocytes (H) according to their age (Ringler & Dabich 1979) and independent of iron injection. By contrast, untreated *b/b* rats have much lower levels for the first six series of measurements, lag in weight and have reticulocytosis. The iron injections produced detectable improvement in Belgrade rats after one week, stabilizing the PCV, Hb and RBC after about a three-fold increase in three or four weeks. By contrast, erythrocyte morphology (not shown) and the parameters reflecting the morphology (MCH at 48 pg, MCV at 8.5 fl and MCHC at 18 g dl^{-1}) were essentially unresponsive to iron injections. Although MCHC exhibited an upward trend, multiple regression analysis indicated that the dependency on age was significant ($P = 0.0005$), but the dependency on injection was not ($P = 0.09$). Injected *b/b* rats also exhibited improved weight gain, but still lagged behind their normal littermates; by eight weeks their reticulocytosis diminished, reflecting the improvement in PCV, Hb and RBC.

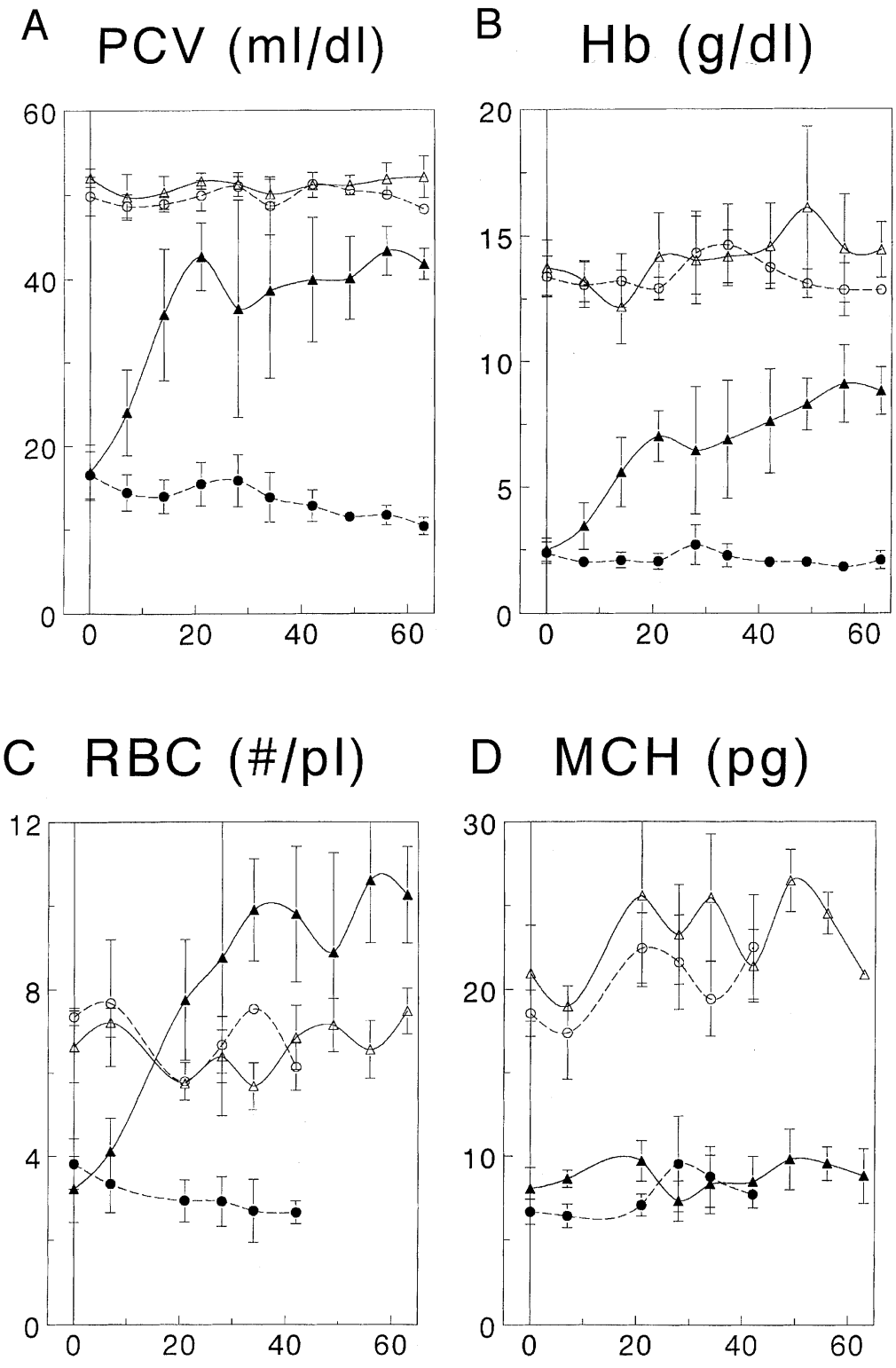

Figure 1. Response of three month old Belgrade rats and littermate phenotypically normal controls to weekly iron injections. Data were fitted by cubic splines; error bars indicate ±1 standard deviation. A few points are missing due to Coulter counter failure. Open symbols represent *+/b* rats; solid symbols represent *b/b* rats. Circles with dashed lines indicate untreated animals; while triangles with solid lines indicate rats that received iron IM.

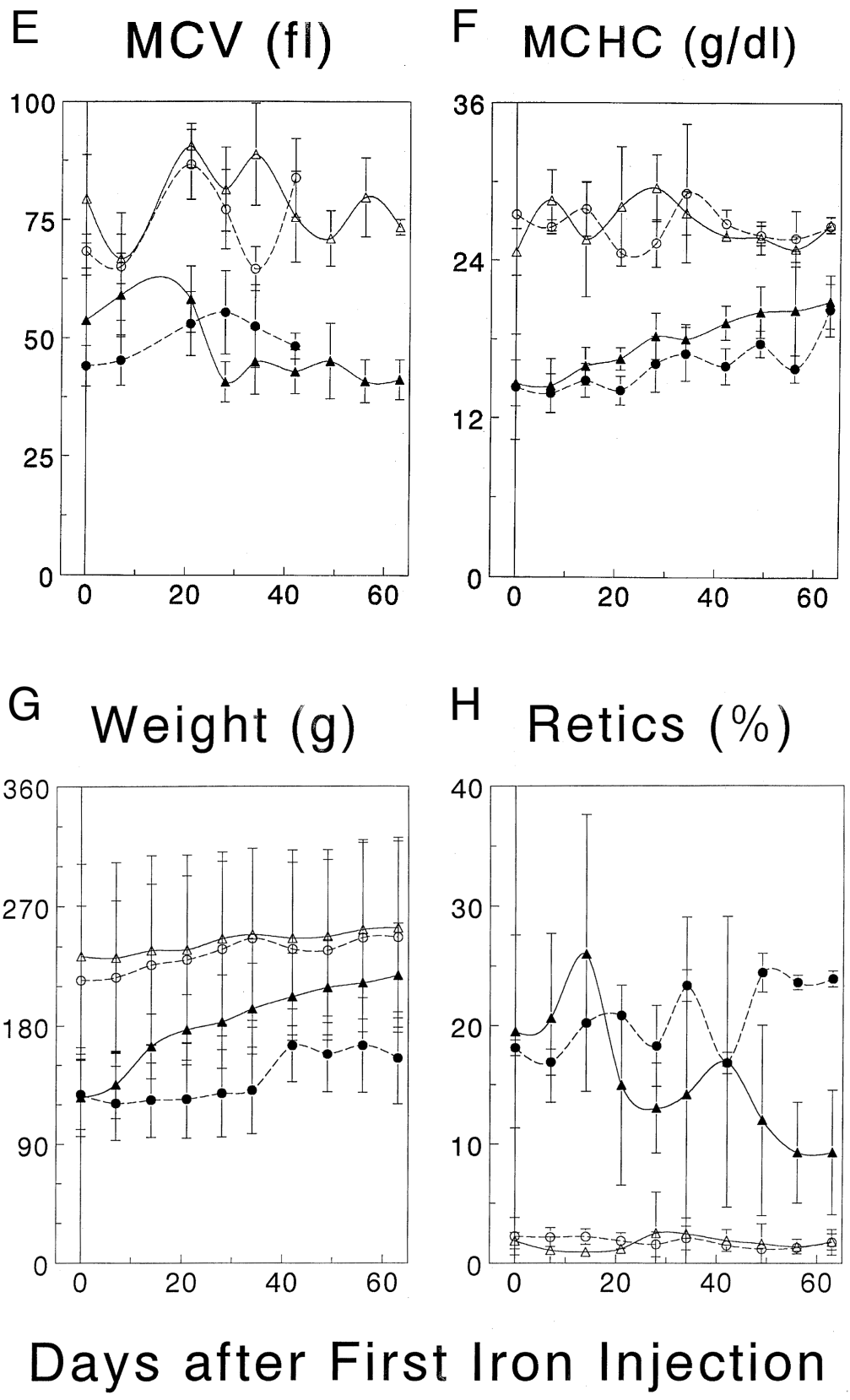

Figure 1. Continued

Female *b/b* rats were fecund after receiving iron injections; by contrast, untreated *b/b* females were infertile, miscarried, devoured their pups, failed to nurse their young, or the pups simply failed to thrive. Remarkably, *b/b* offspring of iron-supplemented *b/b* females had higher RBC counts than *b/b* offspring of unsupplemented +/*b* females. This distinction was maintained until at least six months after birth, suggesting that substantial iron stores were obtained via the placenta and maternal milk.

Dietary iron

Two special diets (Teklad mouse breeder diet and Teklad TD77450) yielded marked improvement in hematological status of *b/b* rats when compared to standard rat chows (such as Purina's or Agway CRM 2000) or even other breeder diets (like Agway CRM 3000 or 3200). Since effective diets had similar quantities of iron to less effective diets, we tested iron bioavailability by adding iron in different forms to Agway CRM 3200 to double the quantity of iron present from 130 to 260 mg kg^{-1}. Three forms of added iron are compared for their effects on six month old rats in Figure 2. The rats were bled on day 0 (just before diet supplementation) and at approximately weekly intervals thereafter. The responses of the animals are shown in Figure 2 in terms of hematological data and growth.

Measurements for +/*b* rats again appeared normal according to their age (Ringler & Dabich 1979). The values were also unaffected by altering the dietary iron. After iron supplementation with $Fe(NH_4)_2(SO_4)_2$ or $FeSO_4$ *b/b* rats respond with increased PCV (A and B), Hb (C and D) and RBC (E and F – eventually exceeding normal controls for RBC), and improved growth (M and N). Despite these improvements, *b/b* RBC morphology and the parameters that are indicative of morphology (MCH – G and H; MCV – I and J; and MCHC – K and L) remain hypochromic and microcytic while reticulocytosis (O and P) is only slightly decreased.

The *b/b* data in Figure 2 were subjected to statistical analyses to ascertain which alterations were significant. When *b/b* rats received unsupplemented CRM 3200, their PCV, Hb and RBC declined significantly ($P = 0.002$, $< 10^{-6}$ and 0.002, by regression) over the two months shown. They also exhibited other indications of severe anemia, poor growth and reticulocytosis. Belgrade rats responded to adding ferric citrate to the diet with increased PCV and RBC ($P = 0.03$ and 0.0001, respectively). Although the difference from no iron addition was significant ($P = 0.00005$ and 0.00001 by analysis of variance), there was no more improvement over another two months and this response is the best that we have seen to ferric iron. By comparison, $Fe(NH_4)_2(SO_4)_2$ led to a highly significant increase in PCV, Hb and RBC ($P = 0.00001$, 0.004 and $< 10^{-6}$ by regression) which differed from no iron addition even more significantly ($P < 10^{-6}$ for all three by analysis of variance). Similarly, ferrous sulfate also increased PCV, Hb and RBC ($P < 10^{-6}$, 5×10^{-6} and $< 10^{-6}$ by regression) and differed from no iron addition with high confidence ($P < 10^{-6}$ for all three by analysis of variance). Thus the response of *b/b* rats to ferrous iron in the diet was much greater than the response to ferric iron. The derived parameters MCH, MCV and MCHC were lower for *b/b* rats than for their normal littermates, but usually appeared unresponsive to dietary iron addition except that Belgrade MCV for ferric citrate supplementation exhibited a decrease that bordered on significant ($P = 0.05$).

Subsequent dietary experiments repeated several of the above iron supplements to examine reproducibility, and compared additional supplements in order to test which factors were critical to the improvement. Data from these experiments are summarized in Table 1 by analyzing the slopes of the PCV data up to the time when it stabilizes (usually < 63 days). Experiment 4 is the same experiment represented in Figure 2. Data were analyzed by regression and analysis of variance. Iron carbonate is largely in the ferric form according to the supplier. Belgrade rats respond well to ferrous iron supplementation but poorly or not at all to ferric iron added to the diet. As was the case for iron injections, dietary iron improves *b/b* rats' health without having much impact on erythrocyte morphology. Whether the formulation is powdered or kibbled appears to be irrelevant.

Tissue iron

Sladic-Simic *et al.* (1969) initially described *b/b* rats as having decreased tissue iron and splenomegaly. It was of interest to re-examine these issues by comparing untreated rats to those on iron supplementations. Figure 3 depicts iron density and total iron after necropsy for five tissues from selected rats representing five experimental groups. Belgrade rats had splenomegaly (spleen weight = 1.9 ± 1.0 g versus 0.35 ± 0.09 g for +/*b* rats [means±standard deviation]). The size of the spleen was not affected significantly by iron supplementation over the terms of the experiments but chronic dietary supplementation led to a decrease for *b/b* spleens which still remained larger than normal (not shown). In the

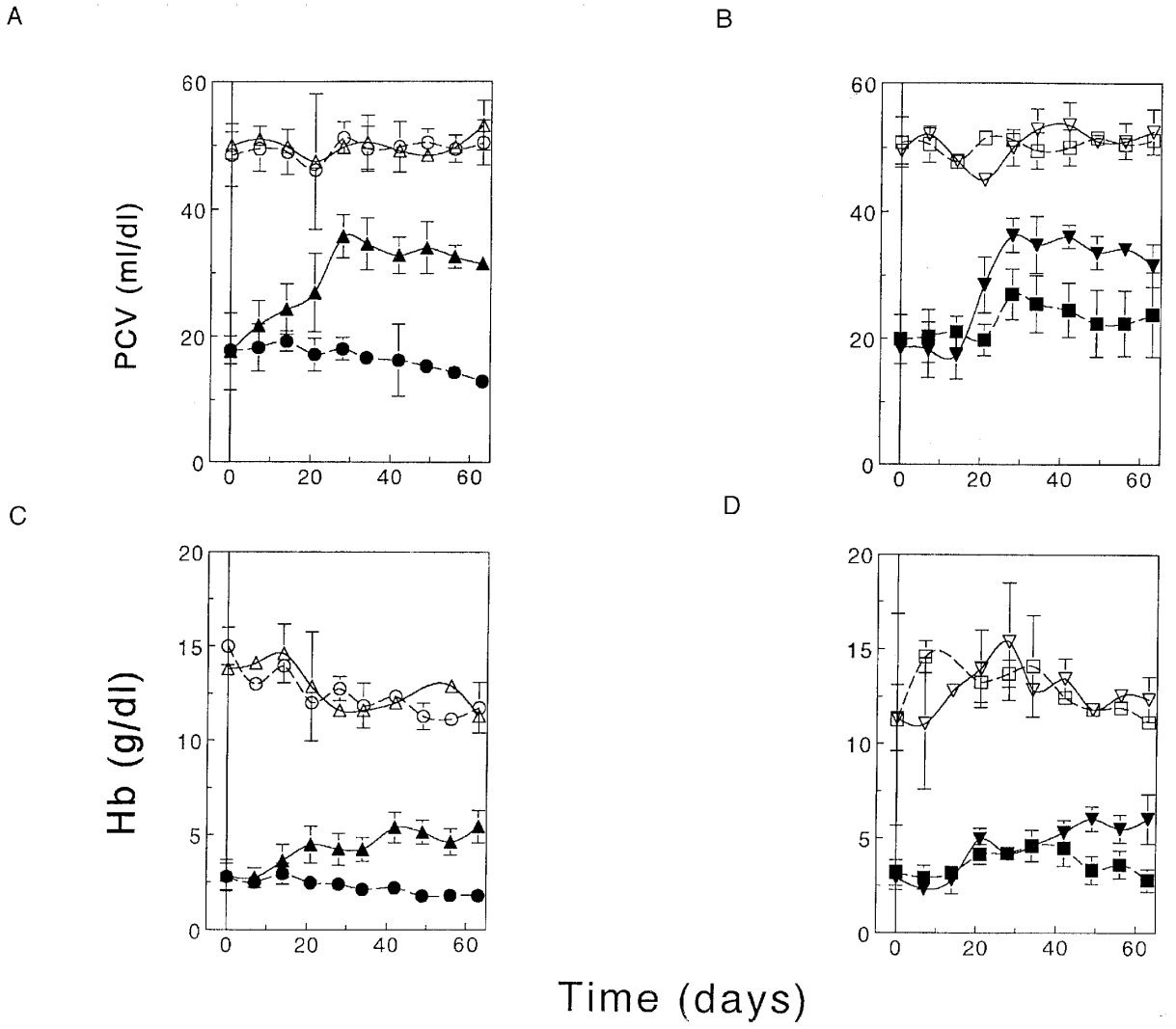

Figure 2. Response of five and a half month old Belgrade rats and littermate phenotypically normal controls to dietary iron supplementation. Data were fitted by cubic splines; error bars indicate ±1 standard deviation. A few points are missing due to Coulter counter failure or other technical problems. Open symbols represent +/*b* rats; solid symbols represent *b/b* rats. The diet was unsupplemented Agway CRM 3200 with 130 mg kg^{-1} of iron present largely as iron carbonate (circles)or the same diet with iron content doubled by adding $Fe(NH_4)_2(SO_4)_2$ (triangles) or ferric citrate (squares)or $FeSO_4$ (inverted triangles).

absence of treatment Belgrade tissue iron density was considerably diminished compared to normal in liver and spleen but relatively unaffected in kidney, heart and brain. Weekly iron injections increased *b/b* liver iron density and total iron above normal (unsupplemented) values, but dietary iron had a more modest effect. Splenic stores for *b/b* rats were relatively unaffected by iron treatments except that total iron for iron injected animals rivaled untreated +/*b* values due to the Belgrade's splenomegaly. Iron injections led to a significant increase in *b/b* cardiac iron, exceeding not only untreated and dietary $FeSO_4$ treated Belgrade animals, but also increasing the iron levels above those of untreated controls. Iron injections caused iron overload in +/*b* liver, spleen and heart as expected.

Serum iron and TIBC

Serum iron, TIBC and % saturation are indicators of the state of iron repletion in an individual. We therefore examined *b/b* and control +/*b* serum to learn how dietary manipulation of iron bioavailability affected these indicators. The serum iron of

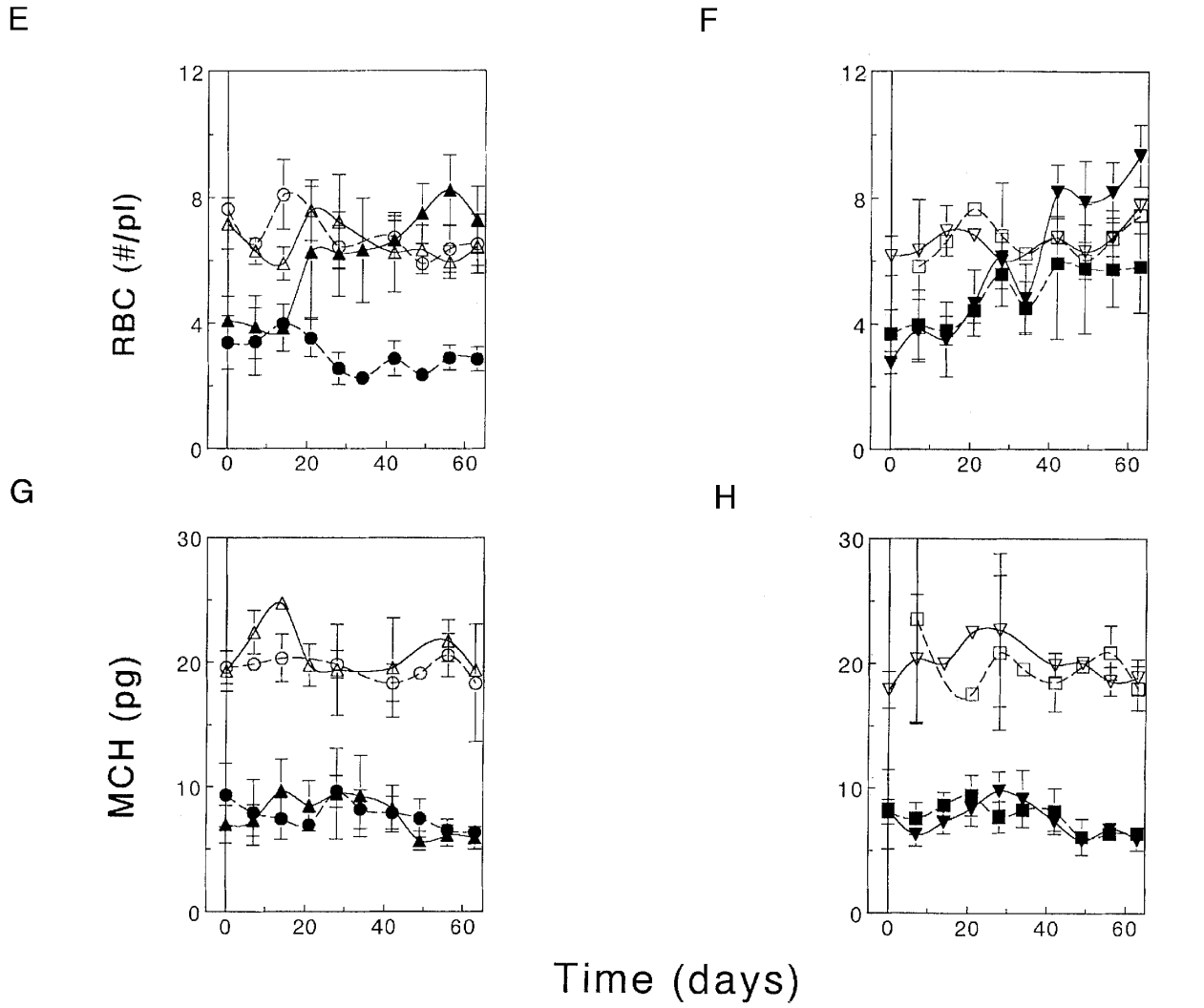

Figure 2. Continued

b/b rats at 406 μg dl^{-1} is nearly double that of +/*b* at 208 (P = 0.01 by anova with the Bonferroni correction for multiple comparisons – also used for the comparisons below); the TIBC shows a similar difference 602 versus 368, respectively (P = 0.0001), but the saturations are not significantly different (67% versus 56, respectively). Except that the differences are less extreme than those previously reported, these values resemble those of past studies (Sladic-Simic *et al.* 1969). Adding $FeSO_4$ to the diet leads to modest, but not significant, increases in +/*b* rats for the serum iron (from 208 to 252) and TIBC (from 368 to 454) while saturation was unaltered (at 56%). Treatment effect on *b/b* rats decreased serum iron from 406 to 372 but the difference was also not significant, while iron supplementation diminished TIBC from 602 to 507 but missed significance (P = 0.08). The two changes increased saturation from 67 to 74% but the change was not significant. Making the comparisons against genotype within the $FeSO_4$ group, one finds that the serum iron difference (from 252 to 372) is still significant (P = 0.04) while the TIBCs (from 454 to 507) and the saturations (from 56% to 74%) are not.

Discussion

Iron injections

Parenteral iron clearly improves the status of *b/b* rats (Figure 1) but leaves the apparent intracellular erythroid iron deficiency unaffected. In Table 2, the data on MCH, MCV and MCHC in Figure 1 are compared to earlier studies. This is because although all

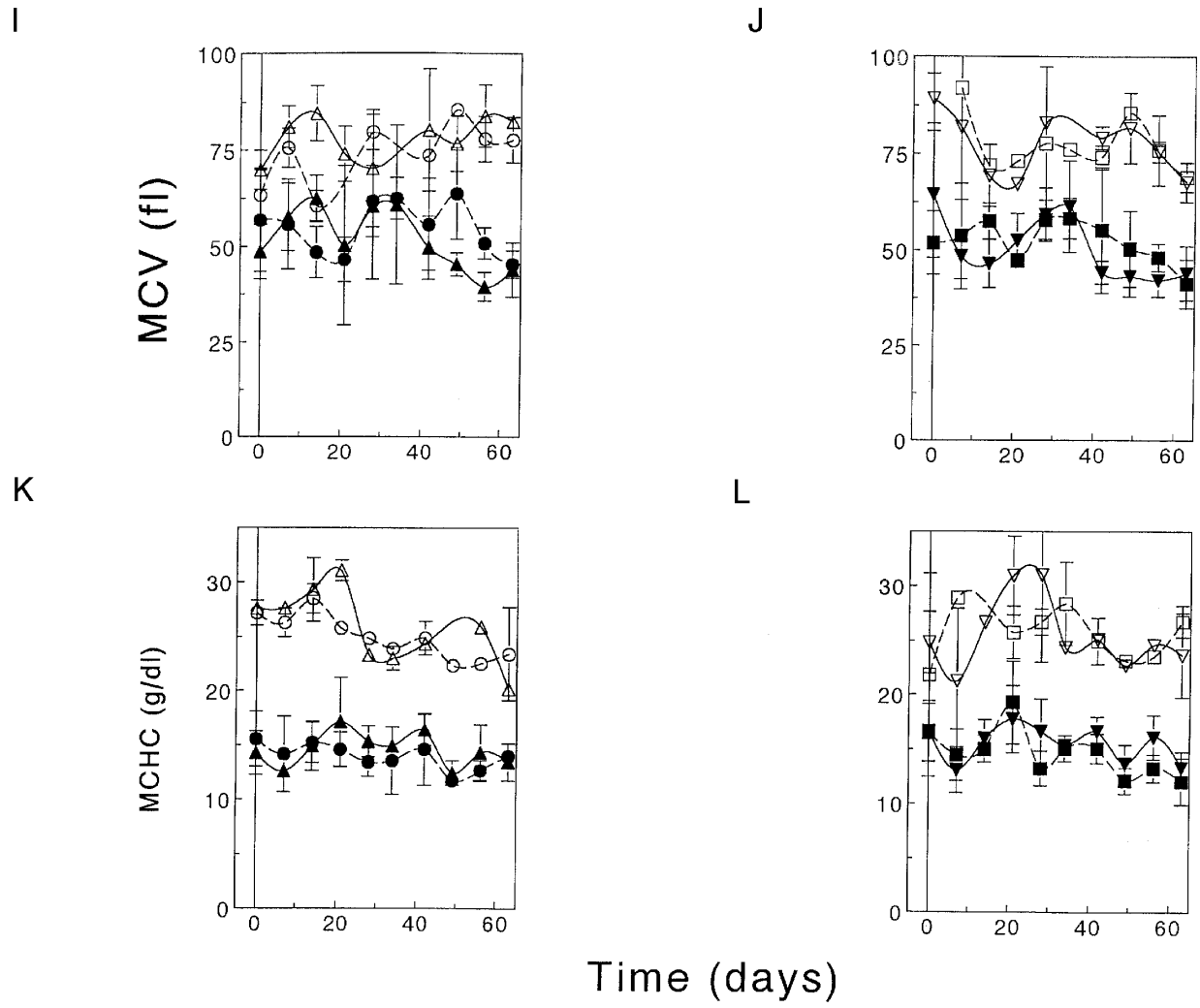

Figure 2. Continued

studies agree that iron treatment improves health, the studies have made varying conclusions about red cell morphology. Some of the variation seen in Table 2 is due to different methods (for example, counting chambers versus automated counters, different windows on the counters) or injection routes and schedules. Nevertheless it is apparent that microcytosis and hypochromia are not eliminated by parenteral iron; at best they may be ameliorated to varying degrees. Because injectable iron leads to an increased production of apparently iron deficient red cells, one must conclude that the underlying defect in iron utilization by reticulocytes is unaltered.

The conclusion that iron treatment is beneficial has important consequences for husbandry of Belgrade rats because iron injections improve the reproductive performance of males considerably and convert females from essentially no fecundity to satisfactory for mating. We have been reluctant, however, to rely heavily on iron injections because an overload of tissue iron develops (Figure 3) given the inability to excrete excess iron and because reticulocytosis decreases, rendering iron incorporation studies more difficult. The increase in erythropoiesis also supports the possibility that iron uptake by the GI tract is insufficient; putting more iron directly available in the circulation alleviates the insufficiency. This insufficiency could be due to an unusual demand for iron, poor gastrointestinal uptake or some combination of the two factors.

Bannerman and Hoke (personal comunication from Hoke) were unsurprised at the failure of *b/b* rats to respond to IP (intraperitoneal) injections of iron given the elevated serum iron (Sladic-Simic *et al.* 1969). We found that this situation is not a nonresponse but rather a slower response exhibited by *b/b*

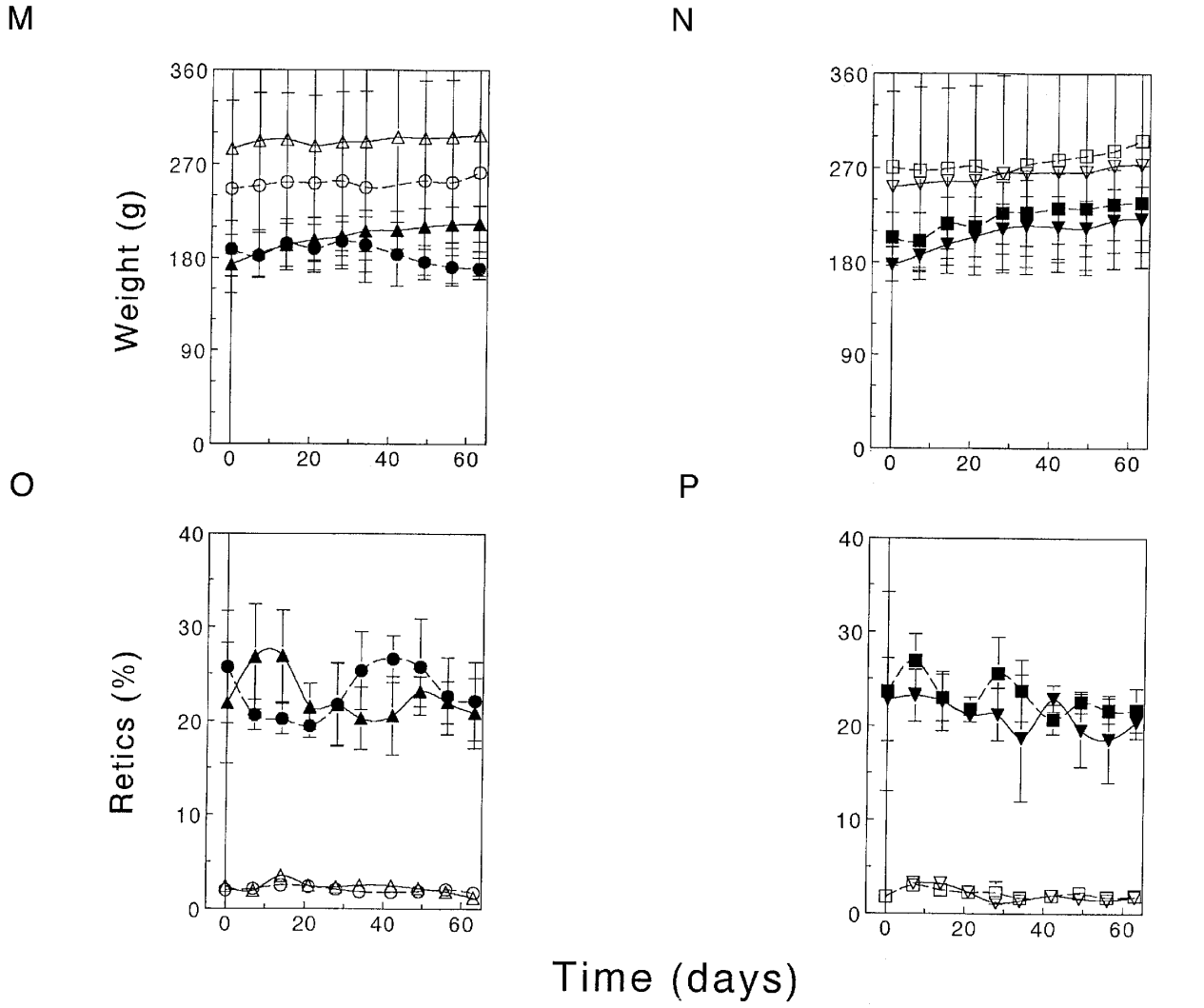

Figure 2. Continued

rats when receiving IP iron (not shown). Ordinarily one expects IP injections to deliver their ingredients as fast or faster than IM. In addition one does not usually expect to monitor differences in impact on a weekly time frame. These considerations suggest strongly that IM and IP iron go to different depots in *b/b* rats, with the depot for IM iron having a more effective bioavailability.

Dietary iron

The observations shown in Figure 2 and Table 1 indicate that dietary ferrous iron supplementation is more effective for Belgrade rats than ferric iron, independent of the associated anion. While this conclusion has been supported for normal circumstances in the past (e.g. Conrad 1987), the present paper is the first demonstration of this nature for this mutant in iron metabolism. Although ferric citrate appears to yield a partial response (Figure 2), it is far less effective. One could argue that adding the iron salt to a diet that already contained some (ferric) iron is actually demonstrating a beneficial effect of having both ferrous and ferric iron present. This argument could be tested by adding various forms of iron to an iron deficient diet but the fact that iron is present as $Fe(NH_4)_2(SO_4)_2$ in Teklad TD77450 (R. Rose, Harlan-Teklad, personal communication) supports the interpretation that ferrous iron is more readily bioavailable to Belgrade rats. It also suggests that the iron citrate in Teklad Mouse Breeder Chow is mostly ferrous. The ability of dietary iron manipulation to improve the RBC count with little effect on the quality of the microcytic,

Table 1. Dietary iron supplementation for Belgrade rats

Expt	Iron supplement	Slope PCV (ml day dl^{-1})	*P* (regression)	versus none	*P* (anova)
4	None*	–0.07	0.002	NA	NA
4	Ferric citrate	0.04	0.03	yes	5×10^{-5}
4	$Fe(NH_4)_2(SO_4)_2$	0.30	1×10^{-5}	yes	$<1 \times 10^{-6}$
4	$FeSO_4$	0.37	$< 1 \times 10^{-6}$	yes	$<1 \times 10^{-6}$
5	None*	–0.12	0.009	NA	NA
5	$Fe(NH_4)_2(SO_4)_2$	–0.002	0.96	no	0.07
6	None*	–0.10	0.40‡	NA	NA
6	Iron carbonate	–0.05	0.14‡	no	0.76
7	None†	–0.03	0.61‡	NA	NA
7	$FeSO_4$	0.08	0.003	yes	0.006
9	None*	–0.11	0.38‡	NA	NA
9	None†	–0.09	0.46‡	no	0.93
10	None*	–0.07	0.06‡	NA	NA
10	$FeSO_4$	0.18	0.02	yes	0.004

NA = Not applicable.
* Agway CRM 3000 diet.
† Agway CRM 3200 diet (differs from 3000 only in the form – 3200 is powdered whereas 3000 is kibbled).
‡ Lack of significance may reflect the limited number of rats and days analyzed or variations in age of the rats at the start of the experiment. Ages were 5½ months for experiment 4; 7 months for 5; 4 months for 6; 9½ months for 7; 9 months for 9; and 1½ months for 10.

hypochromic cells provides investigators with a means of improving the husbandry of this important animal model in iron metabolism without modifying the underlying defect. Because we have seen evidence of iron overload in +/*b* rats maintained for over one year by doubling the iron in Agway CRM 3200 from 130 to 260 mg kg^{-1} with $FeSO_4$ (data not shown), we now routinely add 65 mg kg^{-1} of $FeSO_4$ to CRM 3200.

Metabolic iron depots

Tissue iron measurements (Figure 3) provide quantitative confirmation of prior reports (Sladic-Simic *et al.* 1969) that uninjected *b/b* rats lack tissue iron. Because GI iron uptake is usually responsive to tissue iron deficiency (e.g. Muir & Hopfer 1985), one can speculate that the Belgrade rat is defective at some stage in the transduction of the iron deficiency signal to the GI uptake response. This deficiency is alleviated by dietary iron and even converted into overload by iron injection. Data on the TIBC and serum iron agree with the earlier reports that these values are elevated in untreated *b/b* rats. Although the serum iron and percent saturation suggest that GI iron uptake is adequate, the elevated TIBC suggests that the body 'iron-stat' detects an iron deficiency. The overall improvement of *b/b* rats on dietary ferrous iron supplementation occurs without accompanying iron overload. The accompanying decrease in TIBC suggests that the body 'iron-stat' senses relief of the iron deficiency. It is thus tempting to speculate that the apparent iron deficiency state of the Belgrade rat is recognized by the detection system but that this signal is not transduced properly to lead to a rise in GI iron uptake. If so, then ferrous iron supplementation at least partially bypasses the failure to tune GI iron uptake to match the state of iron stores. The Belgrade mutation could resemble that in hereditary hemochromatosis where the 'iron-stat' is also not working properly, but differ from hereditary hemochromatosis because, in it, GI iron uptake is always set on high, while in the Belgrade rat GI iron uptake is always set on low. While this manuscript was undergoing review, Oates & Morgan (1996) reported studies of GI iron uptake, transfer and absorption by *b/b* and control rats. Belgrade rats exhibited diminished uptake of both ferric and ferrous iron; remarkably and unlike the normal controls, the diminished uptake was relatively unresponsive to alteration of the iron status of the *b/b* rat. Oates & Morgan (1996) also conclude

Table 2. Studies of MCH, MCV and MCHC of Belgrade rats after iron injections

Study	MCH (pg)		MCV (fl)		MCHC (g dl^{-1})	
	Untreated	Fe-treated	Untreated	Fe-treated	Untreated	Fe-treated
Present	7.6	8.5	49	48	15.7	18.0
1966*	5	5	29	20	17	24
1989†	8.0	10.6	44	41	18	27
1990‡	4.9	6.9	24	31	20	22
1991§	8.0	10.8	43	39	19	27
1995¶	7.7	14.8	46	57	16.4	25.4

* Values taken from Sladic-Simic *et al.* (1966); 0.5 mg of iron–dextran injected IM daily.
† Values taken from Pavlovic-Kentera *et al.* (1989); 0.5 mg of iron–dextran injected IM daily.
‡ Values calculated from Kellar *et al.* (1990); 0.5 mg of iron–dextran injected IM daily for 10 days then every other day.
§ Values calculated from Rolovic *et al.* (1991); 3.5 mg of iron–dextran injected IM weekly.
¶ Values taken from Ivanovic *et al.* (1995); 3.5 mg of iron–dextran injected IP weekly.

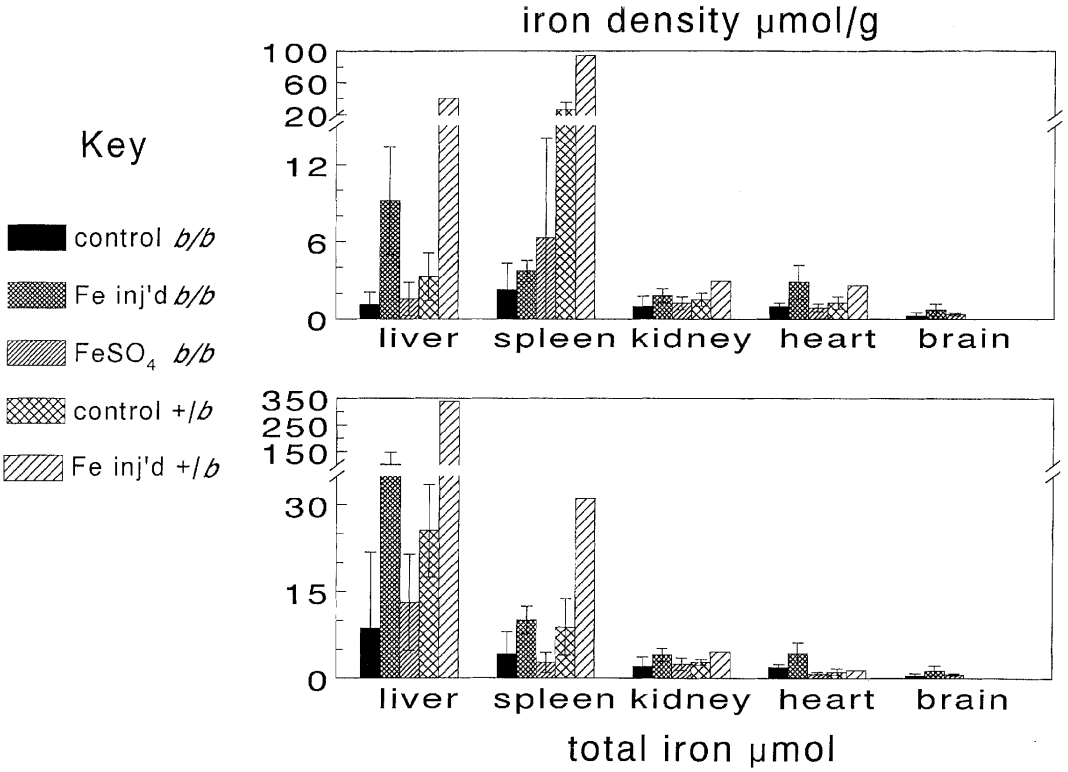

Figure 3. Tissue iron after various iron supplementation regimes. Data from three groups of *b/b* rats (untreated, iron injected and those fed CRM 3200 + $FeSO_4$) and two groups of +/*b* rats (untreated and iron injected) are graphed.

that the defect in iron uptake is not due to a failure to reduce ferric iron, but ferrous iron absorption is not so severely affected by the Belgrade mutation as ferric iron absorption. It will be of interest to learn why dietary ferrous iron supplementation improves the husbandry of Belgrade rats in view of their observations.

Acknowledgements

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