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Iron Dissociates from the NaFeEDTA Complex Prior to or during Intestinal Absorption in Rats

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Sodium iron ethylenediaminetetraacetate (NaFeEDTA) has superior iron bioavailability especially in foods containing iron absorption inhibitors. However, mechanisms involved in the absorption and subsequent partitioning of iron complexed with EDTA are poorly understood. Our objectives were to compare retention and tissue distribution of iron administered to rats either as $FeSO_4$ or NaFeEDTA, either orally (OR) or subcutaneously (SC). Weanling rats were fed semipurified diets supplemented with either $FeSO_4$ or NaFeEDTA for 7 days. They were then given a meal containing ^{59}Fe-labeled $FeSO_4$ or NaFeEDTA, or they were injected SC with these two forms of radiolabeled Fe. ^{59}Fe retention was measured by whole body counting. Urine was collected and counted at 24 h intervals throughout the counting period. Tissue samples were analyzed for nonheme iron and ^{59}Fe activity. Absorption of iron from $FeSO_4$ or NaFeEDTA was similar (57.7 and 53.4%, respectively). Seventy-seven percent of the injected Na^{59}FeEDTA was excreted in the urine within 24 h, whereas only 0.5, 0.8, and 1.4% of the injected $^{59}FeSO_4$, oral $^{59}FeSO_4$, and oral Na^{59}FeEDTA, respectively, was excreted in the urine. The nonheme iron content was lower in the liver and spleen, by 56.8 and 28.4%, respectively, among rats consuming the NaFeEDTA diet as compared to rats fed $FeSO_4$. We conclude that iron is dissociated from EDTA prior to or during intestinal absorption and that some fraction of the dissociated EDTA is absorbed separately from the iron.

KEYWORDS: NaFeEDTA; iron fortification; iron retention; iron distribution; iron excretion; rat

INTRODUCTION

Iron found in food may be classified as either heme iron or nonheme iron. Nonheme iron, found in both animal- and plant-based foods, comprises the majority of dietary iron. The bioavailability of nonheme iron varies greatly according to the composition of a meal, becoming more bioavailable when consumed with meat or ascorbic acid and less so in the presence of iron absorption inhibitors such as phytic acid and polyphenolic compounds (*1*−*3*). Presumably, iron absorption inhibitors form complexes with nonheme iron in the intestinal lumen of the gastrointestinal tract, which either reduces the solubility of iron or limits the accessibility of iron at the site of absorption, thereby ultimately preventing the iron from entering intestinal epithelial cells (*4*). This poor bioavailability of dietary nonheme iron is considered a major factor contributing to iron deficiency (ID), which is one of the most prevalent micronutrient deficiency problems, affecting one-third of the world's population (*5*).

One of the intervention methods for ID is iron fortification. The food vehicle of choice may be plant-based foods that are the primary dietary staples in developing countries or condiments that are regularly consumed. Various forms of iron ranging from iron salts to iron chelates to elemental iron powders have been approved as iron sources for food fortification (*4*, *6*). Because many of the fortificants enter the gut as inorganic ionic iron, their efficacy may still be negatively influenced by the presence of dietary iron absorption inhibitors (*7*). Finding a form of iron whose bioavailability is less affected by other dietary components has become an urgent quest. In the past several decades, there has been considerable interest in using sodium iron(III) ethylenediaminetetraacetate (NaFeEDTA) for food fortification because of its high stability in long shelf life foods, good solubility at low to near neutral pH environments, and superior iron bioavailability in foods containing iron absorption inhibitors as compared with other fortificants such as ferrous sulfate ($FeSO_4$) (*8*−*12*).

EDTA forms stable hexacoordinated chelates with various metal ions. EDTA derivatives such as Na_2EDTA and $CaNa_2$-EDTA are approved food additives to protect the color and flavor of food. Human trials in Thailand, South Africa, Guatemala, China, and Vietnam showed that NaFeEDTA is efficacious in combating ID anemia (*13*−*17*). The U.S. Food and Drug Administration has responded to GRAS notices

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submitted by both Kraft Foods and Akzo Nobel Chemicals and acknowledged that NaFeEDTA is GRAS for addition to powdered meal replacements, flavored milk, fruit-flavored beverages, and soy, fish, hoisin, teriyaki, and sweet and sour sauces (*18*). Nonetheless, mechanisms involved in the absorption and subsequent distribution of NaFeEDTA are not fully understood. For example, it has been proposed that iron is split from the EDTA complex in the intestinal lumen before being absorbed based on animal and human studies (*19*−*21*). However, those study designs failed to include any method to bypass digestion; therefore, the results did not eliminate the possibility that some of the FeEDTA chelate could be absorbed intact and the dissociation occurred after absorption. It is important to further investigate whether the chelated iron is released from EDTA prior to, during, or after absorption, and how much EDTA is absorbed separately, because concerns have been raised over whether the absorption of EDTA from food fortified with NaFeEDTA may cause the mobilization and subsequent excretion of other minerals from the body.

The aim of this study was to address the following questions: (i) Does iron disassociate from the EDTA ligand before or after uptake by the enterocytes? (ii) Can iron be utilized by the body if FeEDTA is taken up as an intact complex? (iii) Is iron absorbed from NaFeEDTA distributed in the body similarly as that from $FeSO_4$? We reasoned that by using the rat model to compare the retention and tissue distribution of $^{59}FeSO_4$ or $Na^{59}FeEDTA$, administered either orally (OR) or subcutaneously (SC), we could make inferences on how NaFeEDTA is absorbed at the brush border of the intestinal epithelium. In addition, we compared the level of nonheme iron in tissues of rats fed with NaFeEDTA to those with $FeSO_4$ to elucidate the effect of EDTA on nonheme iron distribution in the body.

MATERIALS AND METHODS

Chemicals. All chemicals were obtained from Sigma Chemicals (St. Louis, MO) or Fisher Scientific (Fair Lawn, NJ) unless stated otherwise. Water used in the preparation of reagents was 18 MΩ ultrapure water. Glassware and utensils were soaked in 10% HCl for no less than 4 h and rinsed with deionized water prior to use. Radiolabeled ferrous sulfate ($^{59}FeSO_4$) and ferric chloride ($^{59}FeCl_3$) were prepared immediately before use by spiking a solution of unlabeled $FeSO_4$ or $FeCl_3$ with carrier-free ^{59}Fe (Perkin-Elmer, Norwalk, CT) in 0.1 mol/L HCl. For the preparation of radiolabeled NaFeEDTA ($Na^{59}FeEDTA$), $^{59}FeCl_3$ was mixed with a solution of Na_2EDTA at an iron to EDTA molar ratio of 1:1. The pH of this $Na^{59}FeEDTA$ solution was then adjusted to 1 using 0.1 mol/L HCl.

Study Design. Twenty-four weanling, male Sprague−Dawley rats were purchased from Charles River (Willmington, MA). They were housed in a temperature-controlled room on a 12 h dark−light cycle. Stainless steel metabolism cages configured to separate and collect urine and feces were used. Rats were trained for 7 days to accustom them to the cage and diet. During this training period, they were fed a semipurified iron-deficient diet (AIN-93G, Dyets, Inc. Bethlehem, PA) fortified with 35 mg Fe as $FeSO_4$ per kg diet (*22*). The iron-deficient AIN-93G diet (ID basal diet) was previously found to contain 2 mg Fe per kg diet (*23, 24*). The concentration of iron in the acclimation diet, 35 mg Fe/kg diet, has been validated as a level sufficient for growth and for meeting iron requirements without inducing iron loading (*25*).

On day 0 of the experiment, rats were blocked by body weight and randomly assigned to one of four treatment groups of six rats per group. Rats were then fasted overnight before administration of the ^{59}Fe-labeled test dose.

On day 1, 100 μL aliquots of either $^{59}FeSO_4$ or $Na^{59}FeEDTA$ in pH 1 aqueous solution was transferred onto 2 g portions of the ID basal diet and gently mixed to make up test meals containing a total of 70 μg of iron (equivalent to 35 mg Fe/kg diet). The $^{59}FeSO_4$ test meals were fed to group I, and the $Na^{59}FeEDTA$ test meals were fed to group II. All rats consumed the entire 2 g portion they received within 3 h. The remaining two groups (groups III and IV) were given a 60 μL SC injection containing 42 μg of Fe in the form of either $^{59}FeSO_4$ (for group III) or $Na^{59}FeEDTA$ (for group IV). The injection site was on the back, about 2 cm below the head. The size of the injected dose was determined based on the assumption that 100% of the SC injected iron enters the blood stream and therefore is equivalent to 100% absorption from an OR dose. Yeung et al. showed that about 60% of orally administered iron was absorbed by rats in a similar experimental setting (*23*), so an injection containing 60% of the iron in the OR dose was given. The SC injection was chosen as an alternative route to deliver iron to the blood stream via the lymphatic system, bypassing the digestive steps in the gut. The injection is easy to perform, causes minimum stress to the animal, and presumably delivers nearly 100% intact NaFeEDTA complex into the blood. ^{59}Fe activity in rats was determined by a whole-body γ-spectrometer (Tobor Large Sample Gamma Counter, Nuclear Chicago), within 3 h of feeding or injection. Rats in groups I and III were then put back on the acclimation diet, which contained 35 mg Fe as $FeSO_4$/kg diet, while rats in groups II and IV were given a diet containing 35 mg Fe as NaFeEDTA/kg diet.

For the next 10 days (day 2−11), each rat's 24 h urine output was collected. The radioactivity in the urine was also assessed by the γ-counter.

On day 12, rats were killed and samples of the blood, heart, liver, spleen, kidneys, femur bone, and the muscle around the femur were collected. Rats were first anesthetized with isoflurane, and blood samples were obtained by cardiac puncture. Immediately after blood sampling, the breast bones of rats were severed and heart perfusion was performed by slowly injecting physiological saline through the left ventricle of the heart. Specifically, the thoracic cavity was opened by cutting upward bilaterally along the rib wall, exposing the heart. A small incision was made through the apex of the left ventricle, keeping the ventricular septum intact. A perfusion catheter was inserted through the hole in the ventricle into the proximal portion of the aorta and fixed with a hemostat so that the perfusate would enter the circulatory system. A small incision in the right atrium was made to let the perfusate flow out. At least 60 mL of saline was used for each perfusion. Perfusion was deemed complete when perfusate coming out from the right atrium was clear. Organ tissues were removed and immediately counted in the whole-body counter, then accurately weighed, and analyzed for nonheme iron concentration.

Table 1 summarizes the above study design. All rats had free access to their diet and deionized water throughout the study. Rats were observed daily during the whole study for signs of abnormalities. The body weights of the rats were recorded at the beginning of the study and before they were killed. Animal care procedures and experimental protocols were approved by the Institutional Animal Care and Use Committee of the U.S. Plant, Soil and Nutrition Laboratory, USDA/ARS, where this study was carried out.

Calculations of Iron Absorption from Iron Retention. The whole-body ^{59}Fe activity of each rat was determined at time 0 (immediately after the 3 h following administration of the radiolabeled iron) and was used as the initial dose activity for subsequent calculations. The retention of ^{59}Fe in rats was then measured by whole-body counting every 24 h and was expressed as a percentage of the initial dose (the initial retention of ^{59}Fe in all four groups of rats was 100%). Iron absorption was calculated based on the iron retention curve. Specifically, the retention data may be described by exponential functions and used to calculate iron absorption (*26*−*28*). The percentage of absorbed ^{59}Fe was estimated by extrapolating the log-transformed terminal component of the retention curve to time 0. For the two OR groups, the percentage of the actual absorption of ^{59}Fe was estimated by extrapolating the terminal component of the retention curve to time 0. For the two SC groups, however, because we assumed that 100% injected dose was in the blood, hence a 100% initial absorption, an extrapolation of the retention curve represented the percentage of iron that was retained in the body.

Measurement of Iron Content in Tissues. Nonheme iron concentrations in the heart, liver, spleen, kidneys, bone, and muscle samples were determined by the colorimetric method described by Schricker et al. (*29*), with modifications reported by Rhee and Ziprin (*30*) for minimizing the breakdown of heme pigments into nonheme iron. Briefly, about 1 g of tissue was mixed in 10 mL of trichloroacetic acid

Table 1. Experimental Design: Forms and Concentrations of Iron Added to the Rat Diets

group	I	II	III	IV
acclimation period (days −6 to 0)	35 mg Fe (as $FeSO_4$)/kg basal feed (all groups}			
route of administration (day 1)	oral (OR) iron added in basal feed		subcutaneous (SC) iron delivered by a single subcutaneous injection	
dosing (day 1)	$^{59}FeSO_4$	$Na^{59}FeEDTA$	$^{59}FeSO_4$	$Na^{59}FeEDTA$
counting period (days 2–11)	35 mg Fe (as $FeSO_4$)/ kg basal feed	35 mg Fe (as NaFeEDTA)/ kg basal feed	35 mg Fe (as $FeSO_4$)/ kg basal feed	35 mg Fe (as NaFeEDTA)/ kg basal feed
killing (day 12)	harvesting and counting of tissues			

Table 2. Body Weights and Blood Hemoglobin Concentrations

group	I OR $FeSO_4$	II OR NaFeEDTA	III SC $FeSO_4$	IV SC NaFeEDTA	
initial body weight (g)	84.33 (2.14)	84.17 (3.36)	84.67 (2.08)	84.67 (1.99)	$p = 0.999$[b]
final body weight (g)	153.3 (3.17)	158.2 (4.42)	157.2 (4.66)	157.7 (3.16)	$p = 0.813$[b]
hemoglobin[a] (g/L)	163.2 (4.34)	160.6 (4.39)	167.3 (1.80)	167.4 (4.27)	$p = 0.543$[b]

[a] The blood was only collected at the end of the study. [b] One-way ANOVA was used to test significance ($p < 0.05$). No significant differences in the initial body weights, final body weights, or hemoglobin concentrations were detected. All parameters were expressed as means (SEM); $n = 6$.

(20% w/v in 3 mol/L HCl) and a 0.39% sodium nitrite solution. This tissue mixture was incubated at 65 °C for 20 h and then centrifuged at 10000 rpm for 10 min. One milliliter of this supernatant was mixed with 1 mL of hydroxylamine (3% w/v in H_2O) and 1.5 mL of bathophenanthroline disulfonate (BPDS, 0.3 mg/mL in 3 mol/L sodium acetate). Each sample was measured spectrophotometrically at 532 nm against its own blank (supernatant and hydroxylamine only) to minimize any background color interference. Results were expressed as μg nonheme iron per g tissue (wet weight) and were used as indices of rat iron status. The hemoglobin concentrations of the collected blood samples were determined by the cyanmethemoglobin method (*31*).

Statistical Analysis. All statistical analyses were done by using Minitab Release 14 (Minitab Inc., State College, PA). The ^{59}Fe activity in tissues was analyzed by two-way analysis of variance (ANOVA); the effect of treatments on nonheme iron concentrations in rat tissues was analyzed by one-way ANOVA or a two-sample *t*-test. A *p* value of <0.05 was considered significant.

RESULTS

Body Weights and Blood Hemoglobin Concentrations. The mean body weights and blood hemoglobin concentrations are summarized in **Table 2**. There were no significant differences in body weight among the four groups of rats either at the beginning (day 0, $p = 0.999$) or at the end (day 12, $p = 0.813$) of the study. The rats gained an average of 73.1 g during the counting period. No significant differences in hemoglobin level were detected ($p = 0.543$) among the four groups at the end of the study.

Excretion of ^{59}Fe in Urine. The ^{59}Fe activity in each rat's 24 h urine was counted, and the cumulative loss of activity is summarized in **Figure 1**. Generally, the excretion of ^{59}Fe was significantly higher in both groups of rats given NaFeEDTA than those given $FeSO_4$. The most striking ^{59}Fe excretion was from group IV, where rats were injected with $Na^{59}FeEDTA$ SC. Within 24 h of administration, 77% of injected ^{59}Fe was excreted from the rats in this group (**Figure 1a**). This initial sharp increase of iron excretion was not seen in any other group. For example, 1.4% ^{59}Fe was found in the urine after 24 h in group II, where $Na^{59}FeEDTA$ was given orally instead of injected. The excretion of ^{59}Fe was significantly lower when $^{59}FeSO_4$ instead of $Na^{59}FeEDTA$ was administered: Only 0.8 and 0.5% of ^{59}Fe was excreted in the first 24 h from OR $FeSO_4$ and SC $FeSO_4$ groups, respectively (**Figure 1b**).

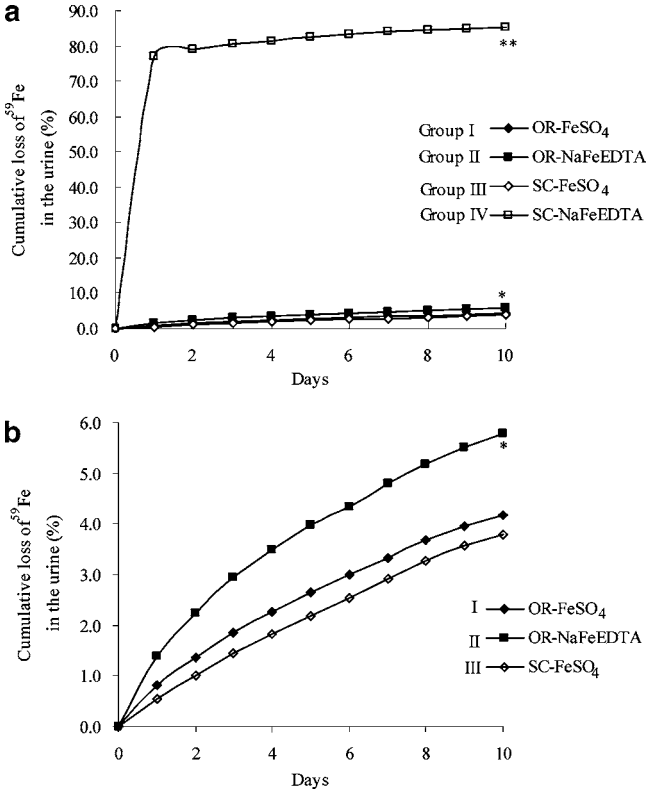

Figure 1. Cumulative ^{59}Fe activity excreted in the urine (expressed as % of dose administered). (**a**) The loss of ^{59}Fe activity in all four groups. OR-$FeSO_4$: (group I), orally administered $FeSO_4$; OR-NaFeEDTA: (group II), orally administered NaFeEDTA; SC-$FeSO_4$: (group III), subcutaneously administered $FeSO_4$; and SC-NaFeEDTA: (group IV), subcutaneously administered NaFeEDTA. (**b**) Cumulative ^{59}Fe excretion in groups I, II, and III only, with the *Y*-axis rescaled. At the end of the study, group IV was significantly higher than the other three (**); group II was significantly higher than groups I and III (*); and there were no significant differences between groups I and III, $n = 6$.

Iron Retention and Absorption. The amount of ^{59}Fe retained in each rat was measured at 24 h intervals after dosing and was expressed as a percentage of the initial dose (**Figure 2**). Each

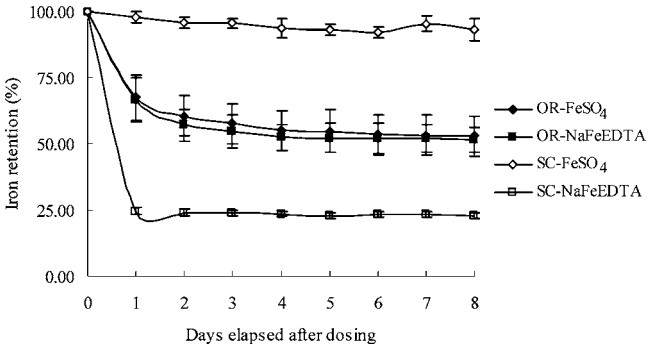

Figure 2. ^{59}Fe retention in the body (expressed as a percentage of the initial dose). This was used to calculate iron absorption.

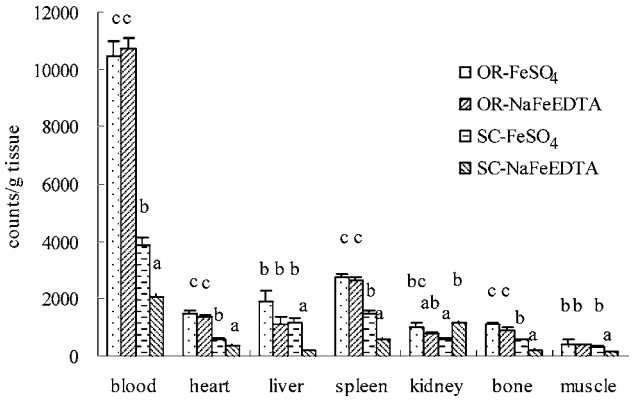

Figure 3. ^{59}Fe activity in selected tissues following OR or SC administration. The blood samples were obtained from the heart puncture. Because heart perfusion was performed, the counting in tissues was assumed blood contamination-free. Within a tissue category, bars not sharing the same letter are significantly different ($p < 0.05$) from each other (two-way ANOVA). Values are mean counts (per gram of tissue) + SEM ($n = 6$).

iron retention curve was roughly separated into two components: an initial rapid decline and a flat, linear pattern, except for the SC $FeSO_4$ curve, which only exhibited the flat pattern. Presumably, the initial decline represented the fecal excretion of unabsorbed ^{59}Fe in the two OR groups and the urinary excretion of injected ^{59}Fe in the SC NaFeEDTA group. The flat, linear pattern represented the gradual loss of absorbed ^{59}Fe (*26*). All four retention curves began to flatten out 2 days after dosing, suggesting that all rats, regardless of the treatment differences, excreted most of the unretained ^{59}Fe within 48 h after treatment. To ensure that all unretained ^{59}Fe had been cleared from the body, only the retention data from day 5 and thereafter were used to calculate the percentage of iron that was truly utilized by the body (iron absorption). The absorption of iron from orally administrated $FeSO_4$ or NaFeEDTA was similar, 57.7% for $FeSO_4$ and 53.4% for NaFeEDTA. For SC injection, on the other hand, the initial absorption was 100% since it was assumed that all the iron entered the blood stream. ^{59}Fe was well-retained (91.0%) in rats injected with $^{59}FeSO_4$, while only 23.2% of ^{59}Fe was retained in rats injected with $Na^{59}FeEDTA$.

^{59}Fe Activity in Tissues. The radioactivity of ^{59}Fe in various tissues is summarized in **Figure 3**. Because cardiovascular perfusion had been performed before tissues were collected, it is unlikely that the radioactivity in the blood affected the counts in various organ tissues in any significant way. When 1 g of each tissue was compared, the blood ^{59}Fe activity was the highest in the OR groups of rats fed $FeSO_4$ and NaFeEDTA. The ^{59}Fe activity was not significantly different between the two OR groups in all tissues tested, despite the difference in the choice of iron compound for the test meal. On the other hand, ^{59}Fe activity in the SC-injected groups was distinctively different from the OR groups. In addition, counts in every tissue of the SC $FeSO_4$ group were significantly higher than the SC NaFeEDTA group. The SC NaFeEDTA group had the lowest ^{59}Fe activity in most of the tissues except for the kidney where ^{59}Fe activity was among the highest.

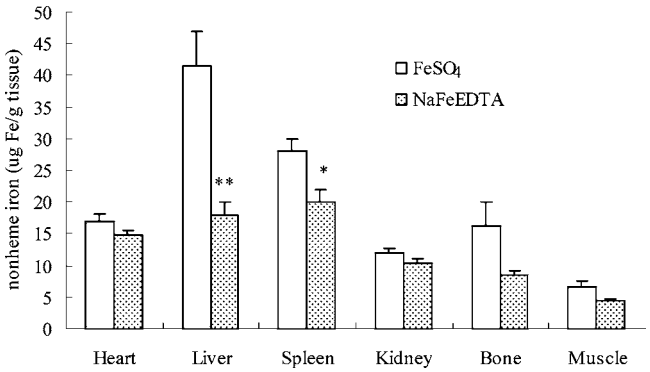

Figure 4. Nonheme iron content in tissues expressed as μg of iron per gram of tissue (wet weight). Asterisks (*) represents significant difference within the tissue category. *$p = 0.006$; **$p = 0.001$. There were no significant differences in the nonheme iron concentrations in the kidneys, bone, and muscles for the two iron sources ($p = 0.067$, 0.063, and 0.078, respectively). Values are means + SEM ($n = 6$).

Nonheme Iron Concentration in Tissues. After counting, tissues were analyzed for nonheme iron content. The results are summarized in **Figure 4**. Because the ^{59}Fe dose was only given once on day 0 at a very low concentration as a tracer, while the 35 mg Fe/kg diet was given throughout the study (for 19 days, including the acclimation period), it is reasonable to assume that nonradiolableled iron from the diet was the major source of nonheme iron in the body. In other words, the form of iron in the diet ($FeSO_4$ vs NaFeEDTA) was the determining factor for nonheme iron, regardless of the routes of administration (OR vs SC). Therefore, groups I and III were combined, and groups II and IV were combined to enhance the power of the test for the effect of diets on the distribution of iron in the body. The only two significant differences were observed in the liver and the spleen, where the total nonheme iron in rats fed NaFeEDTA diet (OR and SC NaFeEDTA groups combined) was 56.8 and 28.4% lower than those fed the $FeSO_4$ diet, respectively.

DISCUSSION

The mean body weights among the four groups of rats did not differ prior to or after the dosing and counting period, indicating that the single dose of SC injection and the types of iron compound in the diet did not affect growth significantly during the 12 days of the study. In addition, the mean final weights, from 153 to 159 g, fall in the normal range of body weight (140−160 g) for 40 day old rats (*32*). The final hemoglobin concentration was not significantly different among the groups and was within the normal range of hemoglobin level for rats. Taken together, it is reassuring that 35 mg of Fe as either $FeSO_4$ or NaFeEDTA supplemented to the ID basal diet achieved similar iron status and the same degree of growth, and no adverse effect on health was observed in rats that underwent the SC injection.

Humans do not appear to possess an effectively regulated mechanism for excreting iron, and normally, the absorbed iron can only be lost slowly. In fact, the daily loss of iron for a healthy man is only 0.025% of total body iron or about 1 mg iron per day (*33*, *34*). Similarly, the iron loss in the urine among the OR-fed rats was less than 1% per day. Although $FeSO_4$

was delivered SC in group III, Fe^{2+} may be oxidized to Fe^{3+} quickly and captured by iron transporting proteins such as transferrin (Tf) in the blood. As a result, SC $FeSO_4$ was efficiently utilized by the body, and the excretion of iron from this group was at the baseline. In contrast, 77% of ^{59}Fe was excreted in the SC NaFeEDTA group, indicating that this portion of iron was still bound to EDTA as intact FeEDTA complex in the blood and, therefore, could not be incorporated into the body. On the other hand, about 20% of ^{59}Fe was retained in the body, suggesting that about one-fifth of iron did dissociate from the EDTA moiety. The specific location where this dissociation occurred is still not clear. One possibility is that apo-transferrin (apo-Tf) in the blood could compete with EDTA for iron. This hypothesis is plausible, because the binding affinity of apo-Tf to Fe^{3+} is about 10^{23} mol/L under physiological conditions (*35*), which is comparable with that of EDTA to Fe^{3+} at neutral pH. FeEDTA passes through the kidneys before being excreted. Therefore, it is also likely that various cations may compete with iron to bind EDTA in the kidneys. The free ionic iron may then be transported back to the body by the divalent metal transporter (DMT-1) located along the nephron tubules (*36*, *37*).

At the end of the study, the OR NaFeEDTA group (group II) also had significantly higher ^{59}Fe excretion in the urine cumulatively than the two $FeSO_4$ groups. It is possible that a small portion of FeEDTA was absorbed intact during digestion, but because most of this intact chelate is not retained in the body, this portion was quickly excreted, causing the small rise of urinary ^{59}Fe after the test meal.

If taken orally, the iron absorption from NaFeEDTA and $FeSO_4$ was similar (**Figure 2**), supporting previous efficacy findings that NaFeEDTA is absorbed as well as $FeSO_4$ (*38*, *39*). The percentage of absorption for the two iron sources, 57.7 and 53.4% for $FeSO_4$ and NaFeEDTA, respectively, was in apparent agreement with previous absorption results of 64.7% for $FeSO_4$ and 49.4% for NaFeEDTA in a similar experimental condition (*23*). We may also infer that iron has to dissociate from EDTA prior to or during uptake by enterocytes, and its absorption is regulated similarly to iron from $FeSO_4$; because if absorbed as an intact chelate, as seen in the injection case, most of FeEDTA would not be utilized in the body and would be excreted in the urine. The retention of SC NaFeEDTA was distinctly different from $FeSO_4$, indicating that adding a strong chelator such as EDTA greatly affects the efficiency of iron uptake by Tf. The activities of retained and excreted ^{59}Fe from **Figures 1** and **2** add up to about 100% of the initial dose, confirming the sensitivity and consistency of these radioactivity assessments.

Most of the ^{59}Fe activity was found in the blood in all four groups (**Figure 3**). This is expected because about 65% of total body iron is incorporated into hemoglobin (*40*). It also shows the importance of a heart perfusion before tissue collection to avoid carry-over radioactivity from the blood in tissues. If administered orally, the radioactivity in all tissues was similar for $FeSO_4$ and NaFeEDTA, again suggesting that iron dissociates from the EDTA complex during digestion and is absorbed and distributed similarly as $FeSO_4$. Because about 80% of activity in group IV was excreted in the urine, it is expected that overall ^{59}Fe remaining in tissues was lower in this group. Indeed, the counts of group IV averaged 77% lower in six of the seven tissue parameters tested. The only exception was in the kidneys where ^{59}Fe was among the highest. This finding could be simply explained as the accumulation of $^{59}FeEDTA$ in the collecting ducts, prior to the excretion. Alternatively, ^{59}Fe may dissociate from the EDTA complex due to the competitive binding of other cations, and is transported by DMT-1 into the interstitial fluid of the kidney. The kidneys are not a common iron storage site, but it is possible that iron is bound to other ligands and is retained there temporarily.

It is puzzling to find lower ^{59}Fe activity in rats injected with $FeSO_4$, especially in the blood, since nearly all ^{59}Fe was retained in the body. One possibility is that the SC-injected iron is distributed differently from the OR iron in the body, and some of the activity had been accumulated in tissues that we did not collect. This hypothesis is unlikely, because the major iron-containing organs such as the liver and spleen (for iron storage) and the blood and bone (for functional iron) have been assessed. It is also unlikely that the injected iron dose overwhelmed Tf, the iron carrier protein in the blood. Unlike an intravenous injection, which delivers a solution directly to the blood, an SC injection allows the incorporation of iron in the blood to occur gradually by going through the lymphatic system first, which reduces the chance of saturating the carrier proteins with a bolus of iron. Moreover, some of the injected iron may nonspecifically bind to serum proteins in addition to Tf. Another hypothesis is that some of the $FeSO_4$ precipitated soon after the injection. This is possible because $FeSO_4$ is less soluble at physiological pH than at pH 1. Once injected in the body, the solution may soon be buffered by the near neutral interstitial fluid and thus may have precipitated in the subcutaneous space or formed complexes with tissue proteins and, therefore, did not reach the circulating blood.

Nonheme iron concentrations in the liver and spleen were significantly lower in the two groups given NaFeEDTA, indicating less iron storage. The rats were not anemic since they all received 35 mg Fe/kg diet and their blood hemoglobin levels were similar to the ones given $FeSO_4$. One possibility is that after the dissociation of iron from NaFeEDTA (either during digestion or in the blood), some of the free EDTA may be absorbed, travel to major iron storage sites such as liver and spleen, and mobilize or redistribute the iron. However, it is not apparent from **Figure 4** which organ or tissue the mobilized iron was deposited. As observed in **Figure 3**, it is possible that iron was redistributed to the kidneys; however, no significant differences were detected between the $FeSO_4$- and NaFeEDTA-fed rats (two-sample *t*-test, $p = 0.067$) in the kidneys. Because this elevated kidney iron was also seen previously (*41*) where a much higher dose (1200 mg Fe/kg diet) of NaFeEDTA was fed to rats for 27 days, it is possible that the dosage of iron in this study (35 mg Fe/kg diet) was not sufficiently high to produce significant kidney iron accumulation.

In conclusion, NaFeEDTA is absorbed as effectively as $FeSO_4$ if administered orally. Iron is dissociated from EDTA prior to or during intestinal absorption. Some fraction of the dissociated EDTA is absorbed separately and may cause the mobilization and redistribution of iron in the body.

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