ABSTRACT Ferritin and soybean meal were reevaluated as dietary treatments of iron deficiency in rats. Isotopes that had been used in the past were avoided because of contemporary knowledge of the physiological and structural complexity of ferritin protein and the solid iron mineral. Rats made anemic by iron-deficient diets were given equivalent amounts of iron as FeSO₄, horse spleen ferritin, baked soybean meal, or soybean meal plus ferritin. Full recovery (89–109%) from anemia and increased tissue iron occurred after 28 d of treatment with any of the iron sources, which contrasts to past bioavailability studies using ⁵⁹Fe-labeled ferritin and generally shorter periods of observation. Cultivar-specific variability was observed in soybean seed soluble iron and ferritin content (1.9–2.0 times the control cultivar, Arksoy), which was apparently heritable. The combined data suggest that manipulating ferritin expression and other soluble components of seed iron in soybeans and possibly other seeds, using Mendelian and biotechnological approaches, could contribute to a sustainable solution to global problems of iron deficiency. J. Nutr. 126: 154–160, 1996.

INDEXING KEY WORDS:
• ferritin • iron deficiency • rats • soybeans

Iron deficiency due to inadequate diet is a global problem affecting an estimated 30% of the population (Baynes and Bothwell 1990). The negative effects of iron-lack anemia on work efficiency have a large economic impact. Perhaps even more profound is the diminution of the rate of normal cognitive development in iron-deficient children (Pollitt 1993), which can have social and economic effects of enormous proportions.

Nature ensures an adequate supply of iron for developing plants and animals by storing iron in ferritin (Brown and Theil 1978, Linder et al. 1972, Lobreaux and Briat 1991, Ragland and Theil 1993, Theil 1980, Theil and Brenner 1981, Theil and Hase 1993), a large protein that reversibly concentrates iron as a solid mineral [Fe₃O₄·(H₂O)₆]; the iron mineral forms in the hollow protein interior, which has a diameter of 8.0 nm (reviewed in Theil 1987, Waldo and Theil 1995). Ferritin is the only protein known that controls the phase transition of ions in solution to a solid phase. Cellular concentrations of iron equivalent to >10¹¹ times the solubility of the free Fe(III) ion can be achieved by ferritin, because the protein plus mineral is very soluble. The importance of ferritin in biology is illustrated by the conservation of amino acid sequence and the quaternary structure in bacteria, plants and animals (Theil 1987, Harrison and Lilley 1990, Waldo and Theil 1995). In addition, the genetic regulation of ferritin expression has features conserved in both animals (Klausner et al. 1993, Theil 1994) and plants (Lescure et al. 1991).

Developmentally regulated changes in ferritin correlate with the accumulation of iron for later use in both plants and animals. For example, iron stored in ferritin of embryonic red blood cells (frogs, chicks, mice and humans) can be shown to be preferentially used for the first generation of adult red cells (Brown and Theil 1978, Theil 1980, Theil and Brenner 1981). In addition, ferritin in the fetal liver at birth is five times that at ~4 d and is used during the period of neonatal nutritional deficit (Linder et al. 1972). In plants, developmental variations in ferritin lead to seed ferritin concentrations that are more than three times

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The phase transition of ions in serum to bone is formally analogous to ferritin function, but bone tissue is a complex of many different macromolecules and cells.

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that in root; for example, after germination cotyledon ferritin concentrations decrease to undetectable levels (Lobreaux and Briat 1991). In addition, sections of maize leaves with immature plastids have ferritin concentrations five to ten times that of the section of the leaf with mature [ferredoxin-containing] chloroplasts (Ragland and Theil 1993, Theil and Hase 1993). Finally, ferritin accumulation in immature soybean nodules is seven times that of mature [leghemoglobin-containing, nitrogen-fixing] nodules (Ragland and Theil 1993).

Because iron stored in ferritin ensures healthy development in plants and animals, ferritin could also be a nutritional source of iron for humans. Earlier studies (Baynes and Bothwell 1990, Derman et al. 1982, Hallberg 1981, Hussain et al. 1965, Laryisse et al. 1975) performed before the developmental features of ferritin regulation were fully appreciated or known concluded that ferritin was a poor source of dietary iron, except possibly when ingested with ascorbate (Derman et al. 1982). In the studies, isotopes of iron added extrinsically to purified ferritin, or intrinsically to animals induced to make excess ferritin, were used to monitor bioavailability of ferritin iron; the assumption made, reasonable at the time, was that the isotopically labeled iron would equilibrate adequately with the solid-phase iron mineral in ferritin. Not well known at the time were the variations in both ferritin protein and iron mineral structures that depended on physiological state and the poor equilibration of added ferric citrate, for example, with the ferritin mineral (see reviews in Harrison and Lilley 1990, Theil 1987, Waldo and Theil 1995). Thus, the method of intrinsically labeling ferritin by giving an animal a 30% increase in the body load of iron, followed by induction of an inflammatory state with nitrogen mustard before injection of the isotope and ferritin purification (Hussain et al. 1965, Laryisse et al. 1975), likely produced a “stress” ferritin not typical of usual foods. In addition, the use of ferritin labeled extrinsically with isotopically labeled ferric citrate (Derman et al. 1982) would measure the availability of only a small fraction of the iron. Even ferritin iron labeled in animals (frogs) undergoing normal development does not fully equilibrate during turnover (Brown and Theil 1978). The current awareness of the conservation of iron regulation of ferritin expression during evolution of animals (Klausner et al. 1993, Theil 1994) and of the use of ferritin as a source of stored iron for embryonic development in plants and animals (Brown and Theil 1978, Lobreaux and Briat 1991, Ragland and Theil 1993, Theil and Brenner 1981, Theil and Hase 1993), coupled with the increased knowledge of the complexity of ferritin protein and mineral structure and function, indicated that a reevaluation of ferritin as a dietary source of iron was timely. If iron in ferritin can be a dietary source of iron, then because ferritin is a natural component of seeds, the potential exists for contributing to a sustainable solution to global iron deficiency through manipulating the ferritin content of seeds.

**MATERIALS AND METHODS**

Newly weaned (21-d-old) male Sprague-Dawley rats were obtained from Harland Industries (Indianapolis, IN) and maintained in an NIH-approved facility; animals suffered no discomfort. All procedures were approved by The Pennsylvania State University Animal Care and Use Committee. Control animals were fed an AIN diet (Borel et al. 1991) with 30 mg Fe/kg for 28 d. A standard AIN diet with 30 mg/kg diet of iron was prepared as described in Borel et al. (1991). Vitamin-free casein and AIN vitamin mixture as described in Borel et al. (1991), were obtained from ICN Biochemicals (Costa Mesa, CA). Cornstarch was purchased from Bordon Feed Service (Columbus, OH), choline from Sigma Chemical (St. Louis, MO) and soybean oil (Hain) from local markets. The AIN-76 mineral mix was described in Borel et al. (1991) and was modified, as described, mainly by using potassium iodide, anhydrous sodium selenide, and the heptahydrate of ferrous sulfate.

Iron-deficient animals [2 wk of consuming an Fe-deficient [2 mg Fe/kg] AIN diet (Borel et al. 1991)] were randomly divided into groups of eight to receive one of the following diets for another 14 d: 1) AIN-Fe deficient; 2) AIN-Fe deficient + FeSO4 [30 mg Fe/kg]; 3) Fe-deficient AIN diet + horse spleen ferritin [13 g Fe/L, 2.7 mL/kg diet, Boehringer Mannheim]; 4) the soybean meal (SBM) diet [Fe-deficient AIN diet substituting 39 g soybean oil for fat, 81 g casein for protein, and 388 g baked [95°C, 1 h] and ground soybeans + 564 g cornstarch for carbohydrate/kg diet, to achieve the AIN values of 10%, 20% and 70% for fat, protein and carbohydrate, respectively]; and 5) the ferritin + SBM diet [the SBM diet plus 2.7 mL of the horse spleen ferritin solution added per kilogram of SBM diet]. Note that the concentration of immunoreactive ferritin in extracts of baked, ground soybeans was the same as for fresh seeds.

Blood was collected from the tail on d 14, 21, and 28 after the animals were given the test diets. Hematocrit [volume fraction] and hemoglobin concentration [g/L of blood] were determined as described in Beard et al. (1988); hemoglobin was measured as the cyanomethemoglobin derivative (see Borel et al. 1991). After 28 d, animals were killed by exsanguination while under anesthesia; livers and spleens were removed, digested and analyzed for total iron by atomic absorption spectrometry [model 5100C, Perkin Elmer, Norwalk, CT; see Borel et al. 1991].

*Analyses of seed ferritin and iron.* Eighteen soybean cultivars, selected for vigor and yield, ob-
tained from the collection at North Carolina State University, were planted in local fields, and the seeds were collected and analyzed. Groups of five seeds of each cultivar were ground in a mortar, extracted with Tris buffer containing 0.1 mol/L NaCl, and the supernatant solution collected as previously described (Ragland and Theil 1993). Equal amounts of protein, measured by the Coomassie Blue assay (Bradford protein kit, Biorad, Hercules, CA), were fractionated by electrophoresis in SDS-acrylamide gels, blotted onto Immobilon membranes and detected with soybean ferritin antiserum coupled with 125I-anti-IgG Fab fragment, followed by autoradiography as described previously (Ragland and Theil 1993). Ferritin was located using a purified ferritin standard (Ragland and Theil 1993); the area of the ferritin band in the autoradiogram for each cultivar extract was measured by densitometry and normalized to the area for ferritin from the reference cultivar (Arksoy), which was selected because it is the progenitor of many widely used commercial cultivars. The Tokyo cultivar had been considered as a breeding stock about 40 y ago because of the very high yield of seeds; an unfavorable oil color led to discontinued use as a breeding stock for oil.

Each cultivar was analyzed at least twice using seed from field-grown plants and in the case of Arksoy, the Tokyo cultivars and its derivative Jackson, seed was examined from plants grown in the phytotron, the greenhouse, and from two sets of field-grown seeds. The mean values were determined after normalizing to the values for standard Arksoy cultivars in each set of analyses.

Soluble seed iron concentration was determined for two to five different samples of each cultivar either colorimetrically or by atomic absorption spectroscopy (Brown and Theil 1978, Ragland and Theil 1993) after digestion in nitric acid. In the case of Arksoy and the Tokyo cultivars, seeds were analyzed from greenhouse plants, phytotron plants, and two years of field crops. There was no detectable difference among the seeds of plants grown at different times in the field, the greenhouse, or the phytotron.

Baking and grinding the seeds for the rat diet had no effect on the integrity of ferritin, as determined by immunoblotting of aqueous extracts. The distribution of iron in the soluble fraction of soybeans was determined by gel filtration on Sephadex G-50 in 20 mmol/L Tris, pH 7.5, 100 mmol/L NaCl buffer. Fractions were analyzed for ferritin by immunoblotting (Ragland and Theil 1993) and for iron as the Fe(II)–1,10-phenanthroline complex (Brown and Theil 1978). The elution volume for monoferric phytate was determined for a solution prepared by mixing equimolar amounts of solutions of phytic acid (Sigma Chemical) and ferric nitrate, followed by adjusting the pH to 7. Immuno-precipitation of iron in the soluble fraction of seeds used a polyclonal rabbit antiserum to soybean seed ferritin (Ragland and Theil, 1993); iron in the precipitates was determined colorimetrically using 1,10-phenanthroline.

**Statistical analysis.** The values are presented as means ± SD. Data were normally distributed, and outliers were examined by using the normality test and boxplot program on Minitab statistical software (MINITAB, State College, PA). Analysis of variance was used to determine whether dietary treatment groups differed significantly with a post-hoc Tukey’s test to establish specific differences in group means. Differences were considered significant when P < 0.05. These analyses were performed using Minitab software using the general linear models procedure.

**RESULTS**

The bioavailability of iron in ferritin and ferritin-containing seeds was tested by comparing the recovery from iron-deficiency anemia among rats fed diets in which the only iron source was FeSO₄ (the standard iron supplement), ferritin or baked soybean meal (SBM). All three iron sources cured the anemia ([Tables 1 and 2]). For example, compared with controls or with iron-deficient animals fed diets supplemented with FeSO₄ (30 mg/kg), rats fed diets containing equivalent amounts of iron as ferritin or SBM had complete (89–100%) recovery in 28 d, based on the hematocrit and hemoglobin concentration ([Table 1]). Hematocrit and hemoglobin of rats maintained on the iron-deficient diets were 41.4% and 35.5% of the control values, respectively. Organ iron was also replenished in rats fed diets with ferritin or baked soybean meal as the iron source ([Table 2]). For example, in rats fed ferritin, the average spleen and liver iron concentrations were 96.4% and 97.2% of the control values, respectively ([Table 2]). Soybean meal was also effective in replenishing organ iron stores: the values were 185% and 313% of values for iron-deficient animals for spleen and liver, respectively, and 65–80% (spleen iron) or 57–64% (liver iron) compared with values of rats fed FeSO₄ or ferritin ([Table 2]). Note that recovery of animals receiving dietary iron supplementation in the form of SBM plus ferritin was indistinguishable from that of animals receiving ferritin supplementation alone, indicating that the SBM does not inhibit iron uptake from ferritin, in contrast to some results previously obtained when seed meal and simple iron salts were mixed in various ways [Davies and Nightingale 1975, Morris and Ellis 1980, Randhawa and Kawatra 1994, Schricker et al. 1982].

The potential for genetic manipulation of soybean seed ferritin content was tested by examining the ferritin protein content in the seed of cultivars previously selected and bred for vigor and yield ([Table 3]). A significantly higher ferritin concentration was observed in two cultivars compared with a standard stock cul-
TABLE 1

Recovery of rats from dietary iron deficiency anemia with iron from purified ferritin (horse spleen) or baked soybean meal (SBM)\(^1,2\)

<table>
<thead>
<tr>
<th>Dietary group</th>
<th>Dietary [Fe], mg/kg</th>
<th>Hemoglobin</th>
<th>Hematocrit</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>14 d</td>
<td>21 d</td>
<td>28 d</td>
</tr>
<tr>
<td>Control</td>
<td>30</td>
<td>145 ± 11.6(^a)</td>
<td>161.5 ± 9.4(^a)</td>
</tr>
<tr>
<td>Iron-deficient</td>
<td>3</td>
<td>68.5 ± 9.2(^b)</td>
<td>58.5 ± 10.0(^c)</td>
</tr>
<tr>
<td>FeSO(_4)</td>
<td>30</td>
<td>68.5 ± 10.9(^b)</td>
<td>149.0 ± 8.0(^a)</td>
</tr>
<tr>
<td>Ferritin</td>
<td>29</td>
<td>66.5 ± 6.1(^b)</td>
<td>122.3 ± 11.4(^b)</td>
</tr>
<tr>
<td>SBM</td>
<td>26</td>
<td>69.3 ± 11.5(^b)</td>
<td>107.3 ± 7.8(^b)</td>
</tr>
<tr>
<td>Ferritin + SBM</td>
<td>55</td>
<td>69.9 ± 10.7(^b)</td>
<td>141.3 ± 11.9(^b)</td>
</tr>
</tbody>
</table>

1 Values are means ± SD, \(n = 8\). Within a column, values not sharing a superscript are significantly different (\(P < 0.05\)).

2 Newly weaned male Sprague-Dawley rats, maintained in an approved facility, were fed either the basal AIN diet (30 mg/kg) (control) or the low iron (3 mg Fe/kg diet) AIN diet for 3 wk. Animals fed the low iron diet were divided randomly into groups of eight to receive the test diet (see Materials and Methods and Borel et al. 1991).

Because seed iron concentration is usually measured on whole seeds, rather than water-soluble and insoluble fractions, variations in seed iron concentration of the ferritin fraction could go unnoticed. Indeed, there was no cultivar-specific variation in the total iron concentration of the soybean cultivars tested. However, in the cultivars with the higher seed ferritin protein concentration, iron concentration was 1.4 ± 0.2 times higher than in the other cultivars in the water-soluble (ferritin-containing) fraction (Table 3).

The soluble iron in the seeds used in the studies described in Tables 1 to 3 was analyzed by gel filtration and immunoprecipitation. As previously observed (Yoshida 1989), much of the iron as well as most of the immunoreactive ferritin, eluted in the void volume, although iron and immunoreactive ferritin could also be observed in other fractions, suggesting interaction with plastid membrane fragments. Thirty percent of the iron or 12 \(\mu g\)/g seed in the soluble fraction was immunoprecipitable by the ferritin antibody, which may be lower than the amount of ferritin iron in the extract, because ferritin could still retain fragments of the plastid membrane that could influence the antibody reaction. Ferric monophosphate eluted behind the void volume when an equimolar amount of phytic acid and ferric nitrate was run through a gel filtration column and thus seemed to contribute little to the soluble iron observed in the soybean seed extracts. Characterization of all the forms of iron in soybean seeds and soluble extracts is beyond the scope of this report.

DISCUSSION

The cultivar-specific differences observed in ferritin and in the iron concentrations of the water-soluble fraction of soybean seeds, in contrast to total seed iron...
concentration, emphasize the importance of fractionating seeds to assess genetic variations in iron content and potential iron availability. Variations in ferritin and water-soluble seed iron, compared with total seed iron, could explain, at least in part, some of the apparent inconsistencies in nutritional iron studies using soybeans, or other seeds, in which only total iron was measured [Davies and Nightingdale 1975, Morris and Ellis 1980, Randhawa and Kawatra 1994, Schricker et al. 1982]. Other differences in experimental results could be caused by differences in how iron isotopes were added to seeds (intrinsically by adding at different stages of plant growth or extrinsically by adding to ground or processed seeds in other studies) [Beard et al., 1988, Davies and Nightingdale 1975, Morris and Ellis 1980, Randhawa and Kawatra 1994, Schricker et al. 1982]. If, for example, intrinsically labeled iron was preferentially in the ferritin fraction and extrinsically labeled iron was preferentially in the hexaferic phy- tate fraction, the results of iron uptake studies could differ substantially. Interestingly, in several recent Mössbauer studies of iron in the hulls and cotyledons of soybean seeds from plants intrinsically labeled with $^{57}$Fe soon after germination and throughout growth, $^{57}$Fe was detected only in ferritin and not ferric phyta- te (Ambe 1994, Ambe et al. 1987). Simple iron salts added as supplements to soybean meal can be bound to phytic acid [inositol hexaphosphate]; hexaferic phyta- te is a relatively nonavailable nutritional source of iron for animals [Davies and Nightingdale 1975, Hallberg 1981, Morris and Ellis 1980, Randhawa and Kawatra 1994, Baynes and Bothwell 1991, Schricker et al. 1982]. The data in Tables 1 and 2 indicate that ferritin iron apparently escapes binding by phytic acid during digestion in rats. In addition, when labeled iron was added early in growth of the soybean plants in other experiments [Beard et al. 1988], an experimental variation in the soybean phytic acid content had no effect on iron uptake in rats and humans. Thus, there are at least two forms of iron in soybeans used in bioavailabil- ity studies: ferric phyta- te and ferritin. Hexaferic phyta- te is water-insoluble [deBoland et al. 1975, Grif- fiths and Thomas 1981, Makower 1970]. Seed ferritin is water soluble [Ragland and Theil 1993]. On the basis of data in Table 3, water-soluble iron was 40% of the total soybean seed iron; the SBM used and seeds tested had normal [Makower 1970] levels of phytic acid (10–11 mg/g alcohol extractable solids).

The distribution of iron among the biochemical components of soybean seeds remains complex. What is clearly known follows: iron incorporated from the roots appears in seeds almost exclusively as ferritin iron [or in polynuclear iron such as phytosiderin, a ferritin derivative that is insoluble [Laulhere et al. 1989]] based on the Mössbauer spectroscopic analysis of soybeans from plants grown hydroponically on $^{57}$Fe medium [Ambe 1994, Ambe et al. 1987]. Iron in soluble extracts of soybean seeds, fractionated on Sephadex G-50 columns, is mainly [90%] eluted in the void volume [Yoshida 1989]. Phytic acid present in soybean seeds, based on the labeling studies, does not contain much of the intrinsic seed iron [Ambe et al. 1987]. The elution of monoferric phyta- te near the void volume of the Sephadex G-50 column, rather than as a small complex, and the absence of iron in equivalent fractions from soluble soybean extracts, confirms the results of Mössbauer spectroscopy of $^{57}$Fe labeled seeds showing little Fe phyta- te [Ambe 1994]. Possibly, the conflicting results obtained in the past for the bioavailability of seed iron [e.g., Beard et al. 1988, Davies and Nightingdale 1975, deBoland et al. 1975, Griffiths and Thomas 1981, reviewed in Hallberg 1981, Morris and Ellis 1980, Randhawa and Kawatra 1994, Schricker et al. 1982] can be explained in part by the apparent structural complexity of ferric phyta- te complexes in soybeans.
Comparison of the results of treating dietary iron deficiency in rats and humans shows the efficacy of using rats for predicting iron bioavailability in humans [Mahoney and Henricks 1984]. Earlier studies on the bioavailability of iron in ferritin, which concluded that ferritin is a poor dietary source of iron (e.g., Baynes and Bothwell 1990, Derman et al. 1982, Hallberg 1981, Hussain et al 1965, Laryisse et al. 1975), relied on the use of isotopes of iron added to ferritin that, based on current knowledge of the complexity of ferritin protein and mineral (Theil 1987), would not likely equilibrate completely with the iron in ferritin. In this study, iron in purified ferritin, iron in baked soybean meal, and purified ferritin added to the baked soybean meal were all able to provide iron to correct dietary iron-deficiency anemia in an animal model system (Tables 1 and 2). Purified ferritin has been shown to be bioavailable. Because at least 15% of the soluble seed iron is in immunoprecipitable ferritin for Tokyo and as much as 90% of total soybean iron is in ferritin-like iron ([soluble ferritin, insoluble ferritin, phytosiderin (Laulhere et al. 1989)] based on Mössbauer studies of 59Fe-labeled seeds [Ambe et al 1987], increasing the amount of ferritin could have a dramatic impact on iron nutrition. Ferritin content is also proposed as the target to modulate because its identity and regulation are better understood than other iron components of the seed. Moreover, the amount of iron per molecule (800–2000 Fe atoms/molecule) indicates that small changes in ferritin expression can have a large effect on iron concentration. Finally, because ferritin is a natural source of iron in development, possible social concerns about additives can be avoided. Thus, the manipulation of the iron and ferritin composition of soybean seeds has the potential for contributing to a sustainable solution to dietary iron deficiency for humans in areas where soybeans are a major food. The genetic variability observed in soybean seed ferritin concentration [Table 2] indicates that classical Mendelian genetics and breeding can be used to increase soybean seed ferritin and iron content. Biotechnological approaches for overexpression of ferritin in seeds can further enhance seed ferritin and iron content. A bonus from such experimental approaches is the general information obtained on the control and expression of genes during soybean seed development, which may be applied to other soybean seed components. Whether the ferritin content of other seeds can be exploited for nutritional benefit remains to be explored.

LITERATURE CITED


