RELATIVE AVAILABILITY OF IRON TO RATS FROM BEEF, SOY PROTEIN AND A BEEF-SOY PROTEIN MIXTURE AS DETERMINED BY IRON REPLETION ASSAY

by

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Male weanling Wistar rats were fed a low-iron basal diet for 3 weeks. The iron depleted rats were then divided into 9 groups according to a randomized block design based on body weight. During the repletion period of 2 weeks, one group was fed the low-iron basal diet. The other eight groups received either the basal diet to which was added 5, 10, 15, 20 or 25 mg iron per kg diet as ferrous sulfate or test source diets formulated to provide a total of 15 mg iron per kg diet from either freeze-dried ground beef, textured defatted soy flour product or a 2.3:1 (w/w) mixture of beef and soy product. All diets were isocaloric and isonitrogenous. The relative biological value (RBV) of iron in the test source diet was calculated as the ratio of the amounts of iron from the reference source (ferrous sulfate) and the test source diet required to give the same response in hemoglobin or hematocrit. The RBVs ± 95% confidence limits, calculated on the basis of final hemoglobin levels and hematocrit values, were respectively: freeze-dried ground beef, 56 ± 7% and 62 ± 7%; fortified textured defatted soy flour product, 81 ± 10% and 79 ± 10%; 2.3:1 (w/w) mixture of freeze-dried ground beef and soy flour product, 65 ± 6% and 68 ± 6%. The RBVs obtained for the iron in beef and for that in the soy flour product suggest that the anemic rat might not be a suitable model for normal man when screening such foods for their available iron. In normal man, the absorption of the iron in beef is comparable to that of inorganic reference iron, while that in textured soy flour is about one third.
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INTRODUCTION

Iron is an essential element for man and other higher forms of life. The major portion of iron in the body is found within the erythrocytes of the plasma as hemoglobin while smaller quantities are present in the liver, spleen and bone marrow as ferritin and hemosiderin, and in muscle tissue as myoglobin. Electron carriers containing iron, such as the cytochromes, are present in all the cells of the body. Together, these iron-containing compounds perform the essential functions of oxygen transport to the tissues and the maintenance of oxidative systems within the tissue cells (Subcommittee on Iron, 1979; Beutler, 1980).

The quantity of body iron is maintained within narrow limits through the regulation of iron uptake by the intestinal mucosa (Subcommittee on Iron, 1979; Beutler, 1980; Narins, 1980). The amount of dietary iron absorbed by the intestinal mucosa is influenced by the chemical form of iron, the composition of the meal and the iron status of the individual (Narins, 1980; Hallberg, 1981b).

In theory, a balanced and varied diet provides a sufficient total amount of iron to more than adequately meet the needs of the population (Narins, 1980). However, it is generally recognized that iron deficiency is a common nutritional problem worldwide. Low availability of dietary iron is considered to be the most important factor responsible for the development of iron deficiency (Monsen et al., 1978).

In recent years there has been a growing trend towards a reduction in red meat consumption by the public. This trend has been motivated by concerns for cost and more recently concerns for nutritional health.
Health and Welfare Canada (1977) published Recommendations for Prevention Programs in Relation to Nutrition and Cardiovascular Disease. Nutritionists have translated these recommendations into practical terms, advising the public to reduce both their fat intake and consumption of animal products.

The reduction of meat consumption has potentially far-reaching nutritional implications. Meat is a very good source of many nutrients, including protein, vitamins of the B complex, iron and zinc (Health and Welfare Canada, 1979). A reduction in meat intake necessitates the compensation of nutrients contained in meat with nutrients from other sources, such as cereals, oilseeds and products made from them. The replacement of meat with low-fat meat substitutes, used as extenders or meat simulators, is a realistic option.

In Canada these meat substitutes, derived largely from plant protein sources, must by law be of a nutrient content equivalent to that of the meat replaced or simulated (Health and Welfare Canada, 1981, Section B.14.073). However, the nutritional availability of the iron in the substituted product is not specified in the regulations, even though iron is generally recognized as being less available from plant than from meat sources (Sabry, 1976).

The objective of this research was to assess the iron availability in a fortified soy protein product and a mixture of beef and the fortified product to determine whether their availabilities compared favorably to that of beef. Iron availability was measured using a bioassay technique, specifically the iron repletion test, using the rat as the assay system.
LITERATURE REVIEW

A. AVAILABILITY OF IRON

The availability or bioavailability of iron from a dietary source, be it a composite meal, single food or iron supplement, describes that portion of the total iron in the source which is absorbed and metabolized to an active species (Mahoney and Hendricks, 1982; O'Dell, 1983).

The amount of iron potentially available from dietary sources depends on a number of complex and interacting factors. The absorption of iron from dietary sources is affected by the digestibility of the iron source, the chemical form of the iron as it occurs in the source, the level of iron in the meal, the iron status of the individual consuming the meal, the presence of other food components in the same meal and the physiological conditions that exist in the digestive tract.

The bioavailability of iron to humans has been extensively reviewed in the literature (Bibeau and Clydesdale, 1976; Bowering et al., 1976; Hallberg, 1981b; Morck and Cook, 1981; Cook, 1983; Morris, 1983) and textbooks (Subcommittee on Iron, 1979; Beutler, 1980; Narins, 1980; Kies, 1982). The various factors that influence the availability of iron will be discussed in subsequent sections.

B. DIETARY IRON SOURCES

The iron in a mixed Western diet is derived from foods of plant and animal origin, from certain additives and from contamination sources (Morck and Cook, 1981). There are two major chemical forms of iron in a mixed diet, heme and nonheme, and each is absorbed by a different mechanism

1. Heme iron

Heme, which contains iron in a porphyrin ring structure, is found in hemoglobin and myoglobin and accounts for about 30-60% of the total iron present in animal tissue (Cook and Monsen, 1976; Schricker et al., 1982). Heme iron from muscle foods enters the intestinal mucosal cell as the heme moiety with the porphyrin ring intact. Within the mucosal cell, the iron-porphyrin complex is catabolized and released ionic iron then enters the same pathways as nonheme iron (Callender et al., 1957; Turnbull et al., 1962; Conrad et al., 1967; Weintraub et al., 1968).

The proportion of heme iron which humans absorb from a mixed North American diet is high in comparison to nonheme iron (Bjorn-Rasmussen et al., 1974). Because of its unique absorptive mechanism and solubility at elevated pH, heme iron is prevented from interacting with other components of the diet (Morck and Cook, 1981). The only dietary factor thought to influence absorption of heme iron is the presence of meat (Hallberg et al., 1979; Hallberg, 1981b; Morris, 1983). The absorption of purified heme iron by human subjects has been observed to increase by a substantial amount when meat is included in the test meal (Martinez-Torres and Layrisse, 1971; Hallberg et al., 1979).

Heme iron provides only 5-10% of the total daily iron intake in mixed Western diets. But as the level of heme iron absorption is high, ranging from 10-25% depending on whether or not meat is included in the meal (Hall-
berg et al., 1979), it provides nearly one third of the iron absorbed each day from mixed diets (Morck and Cook, 1981).

The absorption of heme iron, in comparison to that of nonheme iron, is less affected by the amount of iron administered or by the iron status of the individual at doses in the usual physiological range (Wheby et al., 1970) but at high doses of heme there is a strong correlation with the subject's iron status (Hallberg et al., 1979).

2. Nonheme iron

Nonheme iron, the predominant form of iron in the mixed Western diet, is present in foods such as cereals, vegetables, fruits and eggs and accounts for the remaining portion of iron in animal tissues. Other sources of nonheme iron include compounds added to fortify foods and contaminating iron introduced during the harvesting, processing, cooking or storing of food (Monsen et al., 1978; Hallberg, 1980; Hallberg, 1981a; Morck and Cook, 1981).

The absorption of nonheme iron occurs from a common pool formed by foods ingested in the same meal. The implication of the pool concept is that the absorption of nonheme iron from a meal depends not only on the total amount of dietary nonheme iron but also on the effects of various intraluminal factors, which either enhance or inhibit the availability of iron (Hallberg, 1980; Hallberg, 1981b).

The absorption of nonheme iron is markedly influenced by the iron status of the individual. As body iron stores become depleted more of the nonheme iron from the diet is absorbed; once the iron stores become replenished the level of nonheme iron absorption declines (Monsen et al.,
Absorption of nonheme iron by an individual with adequate iron stores will be about 2% from a meal containing high levels of food components which inhibit the availability of iron. At the other extreme, an iron deficient subject may absorb as much as 20% of nonheme iron from a meal containing an abundance of absorption enhancers (Monsen et al., 1978).

The total amount of iron in the diet has an effect on the proportion of iron that is absorbed; this effect being more pronounced on the assimilation of nonheme iron than that of heme iron. Generally as the level of iron in the diet increases, the proportion of the iron that is absorbed decreases, but the absolute amount increases (Narins, 1980).

As previously mentioned, a small proportion of the nonheme iron of the diets of developed countries is introduced through contamination from the environment (Hallberg, 1980; Hallberg, 1981a; Morck and Cook, 1981). The availability of contaminating iron occurring as insoluble oxides and hydroxides remains to be determined as it does not exchange with soluble radioiron salts used to measure food iron absorption (Hallberg, 1980).

C. CHEMISTRY OF NONHEME IRON

The chemical characteristics of iron such as valence, solubility and chelation have been recognized as being influential in the bioavailability of an iron source (Lee and Clydesdale, 1979a).

Iron has several oxidation states, ranging from Fe$^{+6}$ to Fe$^{-2}$, depending on the chemical environment. The ferric form (Fe$^{+3}$) and the ferrous form (Fe$^{+2}$) are the only states occurring naturally in foods. The iron present in fortification sources may occur in one of three valence states, Fe$^{+3}$, Fe$^{+2}$ or Fe$^{0}$ (Lee and Clydesdale, 1979a).
Ferrous and ferric ions in solution do not occur in the free state, but are hydrated as \( \text{Fe(H}_2\text{O)}\text{\textsubscript{6}}^{+3} \) and \( \text{Fe(H}_2\text{O)}\text{\textsubscript{6}}^{+2} \) in acid and lose protons as the pH is raised to form the corresponding hydroxides in neutral and alkaline solutions. These hydroxides become more insoluble with increasing pH. At neutral pH, in the absence of ligands, ferrous hydroxide has a solubility of about \( 10^{-1}\text{M} \) while that in the ferric form is much less soluble, having a solubility of \( 10^{-16}\text{M} \) (Lee and Clydesdale, 1979a).

Iron in the ferrous state is absorbed more efficiently by man than iron in the ferric state (Brise and Hallberg, 1962). It remains to be determined whether this phenomenon is due to the higher degree of solubility of ferrous iron or to a selective absorptive mechanism (Lee and Clydesdale, 1979a; Nojeim and Clydesdale, 1981).

Many common food components are effective chelating agents or ligands and can prevent the precipitation of iron in the neutral to mildly alkaline environment of the small intestine (Lee and Clydesdale, 1979a), the site at which the majority of dietary iron is absorbed (Wheby et al., 1964). The absorption of chelated iron, however, may be enhanced or inhibited depending on the nature of the specific iron complex formed, which includes its solubility, molecular weight and stability (Monsen and Page, 1978; Lee and Clydesdale, 1979a; Clydesdale, 1982).

Enhanced iron absorption has been observed when such organic acids as ascorbic acid, sugars such as fructose, and amino acids like cysteine are present in the meal. It is postulated that these compounds form soluble low molecular weight, relatively weak chelates with iron. These chelates keep the iron in solution during transit through the neutral to alkaline
environment of the upper small intestine (Monsen et al., 1978; Lee and Clydesdale, 1979a; Morck and Cook, 1981).

Ascorbic acid and cysteine, acting as reducing agents, have been reported to increase iron absorption by reducing ferric iron to the more soluble ferrous form (Lee and Clydesdale, 1979a; Morck and Cook, 1981).

Food components reported to have a negative effect on iron absorption include tannins, EDTA, oxalates, calcium and phosphate salts, phosphoproteins, phytate and carbonates. Various mechanisms have been proposed as the reason these iron-complexing compounds decrease the availability of dietary iron; among the suggestions are the formation of insoluble precipitates or strongly bound soluble complexes with ferric iron at neutral pH (Bibeau and Clydesdale, 1976; Monsen and Page, 1978; Morck and Cook, 1981).

D. EFFECT OF VARIOUS PROTEIN SOURCES ON THE ABSORPTION OF FOOD IRON IN MAN

1. Animal protein

A "meat factor" present in meat, poultry and fish has been shown in humans to raise the rate of nonheme iron absorption from a meal by as much as four times (Cook and Monsen, 1976). As the quantities of these substances in a complex or composite meat increase, iron absorption increases. Neither protein nor animal protein per se enhance iron absorption. While beef, lamb, pork, chicken, liver and fish increase nonheme iron absorption, milk, cheese, and eggs do not increase and may decrease iron absorption (Cook and Monsen, 1976). It was shown years ago by Layrisse et al. (1969) that the absorption of iron from different vegetable foods was markedly increased when they were served together with
meat and fish. Initially, this effect was considered to be due to an effect of amino acids formed during the digestion of meat (Martinez-Torres and Layrisse, 1970). The results of later studies, however, and especially the observation that egg albumen does not enhance dietary iron absorption, in spite of the fact that it has about the same amino acid composition as meat and fish, are not consistent with the initial interpretation (Cook and Monsen, 1976).

Bjorn-Rasmussen and Hallberg (1979) performed a series of studies with humans in an attempt to gain some insight into the mechanism of action of meat on the absorption of nonheme iron. The effect of beef on absorption of food iron was studied in patients with proved gastric achlorhydria to see if the effect was related to the stimulus of the secretion of HCl; the effect of various foods (beef, chicken, fish, thymus and egg albumen) was compared; the effect of cysteine or a mixture of amino acids in amounts and proportions corresponding to the contents of beef was studied; minced beef was boiled with water, and the effects of broth and meat residue were measured separately; the effect of beef meat on the absorption of inorganic ferrous and ferric salts (given alone or together with sodium phytate) was studied. The test meal consisted of a maize porridge that had been extrinsically labelled with $^{59}\text{Fe}$ as ferrous sulfate.

Measurements of iron retention showed the relative enhancement of iron absorption was the same for both normal and achlorhydric subjects. The meat products (beef, fish, chicken and calf thymus) all increased iron absorption to the same extent. Neither egg albumen, cysteine or a water extract of beef did, however, affect the absorption of food iron. Beef
increased the absorption of a solution of inorganic iron given without food only when the iron salt was trivalent or when sodium phytate was added to the solution. It was concluded that meat acts by counteracting luminal factors that inhibit iron absorption; the most probable mechanism for this action being the formation of a luminal carrier which transports the iron to the mucosal cell membrane.

Hallberg et al. (1979) conducted studies on a mixed group of human subjects, which included both normal adults and blood donors, to determine the effect of meat on hemoglobin iron absorption. Testmeal A (meat-free) consisted of two wheat/rye rolls made from unfortified flour (radiolabelled with Hb-$^{59}$Fe), served with margarine, cheese, marmalade, cereal with milk, and coffee. The meat-containing test meal (B) consisted of meal (A) plus two beef patties. Both meals contained 5 mg radiolabelled heme iron. It was found that the addition of meat to the meal increased the percent Hb-$^{59}$Fe absorption from 10.2% in the meat-free meal to 16.0% when meat was included. The mean ratios of Hb-$^{59}$Fe: reference iron (FeSO$_4$) absorption were 0.20 and 0.31 for the meat-free meal and meat-containing meal, respectively. The authors postulated that absorption-enhancing effect of meat on both heme and nonheme iron might be due to stimulation of the digestion of food (by pancreatic or biliary secretions) so that iron in either form is more efficiently released and made available for absorption.

2. Soy protein

Human studies have demonstrated that the addition of soy protein-derived food products to a meal can either inhibit or enhance the absorption of nonheme iron already present in the meal. The effect soy protein
has on dietary nonheme iron absorption largely depends upon the composition of the meal to which it is added, that is, to what extent the meal contains or lacks enhancing or inhibitory food components.

Cook et al. (1981), using an extrinsic tag method with human subjects, demonstrated that soy, as a protein source, depressed dietary nonheme iron absorption. These researchers substituted, in protein-equivalent amounts, a wide range of soy products, including full fat soy flour, textured soy flour and soy isolate, into a semisynthetic egg albumen-based meal. All diets were adjusted with FeCl₃ to provide the same amount of iron. The mean nonheme iron absorption values were observed to fall from 5.5% in the unsubstituted meal to 1.0, 1.9 and 0.4% respectively.

Hallberg and Rossander (1984), in a human study using extrinsic tag methodology, investigated the effect of adding soy protein to a simple Latin American cereal-based meal to see whether the absorption of nonheme iron could be improved. The basal diet, consisting of maize chapattis, black beans and rice, was high in phytic acid and lacked enhancing factors. There was only a marginal improvement in percent absorption of dietary nonheme iron with the addition of soy; the percent iron absorption from the basal diet alone being 3.5% while the addition of defatted soy flour increased the iron absorption to 4.0%. However, this addition of soy tripled the quantity of nonheme iron absorbed from 0.17 to 0.51mg as a result of the high iron content of the soy product.

Studies examining the effect of replacing a portion of meat in a hamburger meal with soy protein product have shown that under such circumstances the absorption of nonheme iron is inhibited (Cook et al.,
1981; Hallberg and Rossander, 1982). The overall resultant reduction in absorption is due to a combination of the inhibitory action of soy and the reduction in the enhancing action of the meat (Morris, 1983).

Cook et al. (1981), in a human study applying the extrinsic labelling technique, found that the percent nonheme iron absorption decreased from 3.2% in the beef protein-based meal to 1.24 and 1.51% when 3:1 and 2:1 ratios (w/w) of meat to unhydrated textured defatted soy flour, respectively, were fed.

Hallberg and Rossander (1982), who extrinsically labelled both the heme and nonheme iron of a hamburger meal, demonstrated that when half of the beef of a hamburger was substituted by a protein-equivalent amount of textured soy flour or defatted soy flour the percent nonheme iron absorption in normal human subjects decreased from 8.4% to 7.2 and 5.2%, respectively. This observed decrease in nonheme iron absorption was attributed to the reduction of beef, an animal protein demonstrated to stimulate iron absorption (Cook and Monsen, 1976; Hallberg et al., 1979). However, the amount of nonheme iron absorbed from the soy containing meals compared favorably to that from the beef-based meal; the high iron content of the soy products being the reason for this similarity. The inhibition observed in this particular study was not alleviated when dephytinized soy was included in the meal. Upon examining the effect of partial substitution of soy for beef protein (1:1) on the amounts of heme and nonheme iron absorbed, it was found that the total amount of iron (heme and nonheme) absorbed from the beef and beef-substituted meals did not differ.
E. EFFECT OF PROCESSING ON THE AVAILABILITY OF MEAT IRON

The results of early studies on the effect of heat processing on the availability of heme iron to humans have been inconsistent. Boiling of intrinsically labelled rabbit hemoglobin for 3 minutes decreased the intestinal absorption of hemoglobin iron from 11 to 7% and from 22 to 12% in normal and iron deficient subjects, respectively (Callender et al., 1957). On the other hand, Turnbull et al. (1962) reported that cooking hemoglobin in a boiling water bath for 15 minutes did not alter the absorption of the hemoglobin iron in iron deficient subjects.

More recently, Schricker et al. (1982) demonstrated that dry heat processing (100°C for 20 min) of fresh ground beef resulted in an increase of 110% in the nonheme iron content of the sample (9.9 μg/g in the raw sample compared to 20.9 μg/g in the cooked). This increase in nonheme iron concentration apparently results from release of iron from the heme complex, possibly through a mechanism involving the oxidative cleavage of the porphyrin ring (Schricker et al., 1982). It seems possible that different cooking methods could result in different proportions of heme and nonheme iron in meat. Since the availabilities of heme and nonheme iron in humans are substantially different, more information on the effects of cooking on heme degradation is needed (Schricker et al., 1982).

The results of rat feeding studies employing hemoglobin repletion methodology have shown that the effect of heat treatment on meat iron availability is variable. Oldham (1941) found that the hemoglobin gain in rats fed with oven-dried meat was higher than for those fed with unheated, vacuum dried meat. A recent study by Jansuittivechakul et al. (1985) investigated the effect of autoclaving, boiling and baking on the
availability of iron in beef in anemic rats. It was found that cooking meat did not significantly affect iron bioavailability compared with uncooked product. Furthermore, the decrease in heme iron in meat due to cooking was not associated with any decrease in bioavailability of meat iron as fed to rats.

F. TECHNIQUES FOR THE DETERMINATION OF IRON AVAILABILITY

1. Chemical Balance

The earliest attempts to measure the quantity of dietary iron available for absorption were made with balance techniques whereby estimates of iron absorption were based on the differences between oral intake and fecal loss of iron (Moore, 1968). The chemical balance technique is the only method that directly measures iron absorption from the whole diet (Hallberg, 1981b). This method, however, is insensitive, imprecise, and time consuming, and it gives no information about iron absorption from different meals (Hallberg, 1981b). The technical errors of the method include the inherent lack of precision in measuring the small difference between oral intake and fecal loss and the impossibility of differentiating between endogenous loss and unabsorbed iron (Moore, 1968).

2. Radioisotopic labelling

Radioisotopic labelling techniques allow the most precise measurements of dietary iron absorption in humans and animals (Narins, 1980). Estimates of iron absorption from a given source are based on the measurement of radioactivity remaining 10 to 20 days following the consumption of food containing a known amount of radioiron (Narins, 1980). Radioiron
absorption may be determined by either measuring erythrocyte incorporation or whole-body retention of radioiron (Narins, 1980). The whole-body retention of radioiron is regarded as being not only reliable and sensitive but indeed the only completely satisfactory quantitative method but is not widely used in human studies due to the expense of the whole-body counter (Narins, 1980). Though less accurate, the measurement of blood radioactivity is the method most extensively used in human studies of iron absorption (Narins, 1980). The errors of this method include the variability of the red blood cells in incorporating the radioiron and in the use of estimates of total blood volumes from height and weight figures (Narins, 1980).

Biosynthetic labelling (intrinsic labelling) was first introduced by Moore and Dubach (1951) and is considered by some to be the most valid approach to measuring absorption of food iron (Narins, 1980). This labelling technique involves the biological incorporation of radioiron into single food items. Vegetable foods are labelled by growing them in hydroponic medium containing radioiron while animal foods or fish are similarly tagged by injecting or feeding radioiron in the months prior to sacrifice (Narins, 1980).

Studies with intrinsically labelled foods demonstrated that iron absorption from individual foods differed markedly (Moore and Dubach, 1951). Also, when two iron-containing foods were fed together, the amount of iron absorbed from each food differed from that when fed separately, thereby demonstrating that a food interaction had taken place (Layrisse et al., 1968). Thus, knowing the iron absorption from single foods does not
provide a valid estimate of the absorption of iron from a whole meal (Hallberg, 1981b). Disadvantages of the intrinsic labelling technique include the time, expense and difficulty involved in the preparation of biosynthetically labelled foods (Narins, 1980). The application of this labelling technique to the estimation of iron absorption from complete meals is limited by the availability of only two radioiron isotopes (Narins, 1980). This type of labelling, however, remains the only one available to study insoluble forms of intrinsic food iron (Narins, 1980).

The development of the extrinsic radioiron tag technique, whereby a radioactive tracer is added to either a complete meal or one of the components of the meal prior to its consumption, has made it possible to obtain an estimate of the absorption of the intrinsic iron from selected single foods and complex meals (Hallberg, 1980; Narins, 1980; Hallberg, 1981b; Consaul and Lee, 1983; Van Campen, 1983). The validity of the method is based on the assumptions that (1) heme iron and nonheme iron from the diet form two separate pools of iron in the gastrointestinal tract; and (2) these dietary iron pools can be independently and simultaneously labelled through the use of two different radioiron isotopes that exchange completely with their respective iron pools. The heme iron pool is labelled with biosynthetically radioiron-labelled hemoglobin and the nonheme pool with an extrinsic inorganic radioiron tracer (Hallberg, 1980; Hallberg, 1981b; Van Campen, 1983). The extrinsic tag technique is not applicable to all food items, nor can it be used in determining the absorption of insoluble forms of iron (Hallberg, 1980; Hallberg, 1981b; Consaul and Lee, 1983; Van Campen, 1983). While the extrinsic labelling
technique offers a convenient alternative to the more specialized, time-consuming techniques of preparing intrinsically labelled foods, it may be associated with an error of 20% when estimating bioavailability of iron (Weaver et al., 1984).

3. In vitro

Various in vitro techniques have been developed to estimate the amount of dietary iron that is potentially available for absorption (Miller and Schricker, 1982). Early methods interpreted available iron to be the measurable ionizable iron in foods. Ionizable iron has been determined as that fraction of the total iron in a food that will react with a complexing agent, such as α, α'-dipyridyl, tripyridyl triazine or bathophenanthroline, to form a chromagen which can be quantitated spectrophotometrically (Narins, 1980; Miller and Schricker, 1982). Determinations of ionizable iron in foods are of little physiological significance, however, since the conditions of the gastrointestinal tract which influence iron availability are not considered (Narasinga-Rao and Prabhavathi, 1978).

Recent in vitro methods have concentrated on simulating gastrointestinal digestion using purified peptic and/or pancreatic enzymes with subsequent measurement of either the soluble or dialyzable iron (Miller and Schricker, 1982; Van Campen, 1983). Miller et al. (1981) has refined the procedure used by previous researchers to better simulate in vivo digestion conditions.

Schricker et al. (1981) compared the in vitro method of Miller et al. (1981) with rat and human extrinsic radioiron tag techniques. Complex meals were used in the comparison. The ability of this in vitro method to
show statistically significant differences between iron availability in the various meals substantially agreed with that of the human in vivo method whereas there was less agreement between the in vitro and rat in vivo methods.

Advantages of in vitro methods include their rapidity and low cost, their reduced variability compared to in vivo methods and the ability to precisely control conditions during the determinations. In vitro methods are viewed as being useful in preliminary studies of iron availability (Schricker et al., 1981). Limitations of the method include the inexact duplications of in vivo conditions and the inability to account for the effects of factors involved in the absorptive process such as active transport and brush border binding proteins (Miller and Schricker, 1982). Given these limitations, in vitro measurement of iron availability must be regarded as a relative rather than an absolute indication of availability (Miller et al., 1981).

4. Animal bioassay

Efforts to develop an appropriate biological assay to assess iron availability were begun years ago when interest arose regarding the fortification of foods with iron (Elvehjem et al., 1933; Smith and Otis, 1937; Freeman and Burrill, 1945). Pla and Fritz (1970) revived efforts in this area by introducing an assay method involving young anemic rats (or chicks) which utilized changes in hemoglobin concentration (or hematocrit level) as the criterion for assessing the availability of the iron source.

Following collaborative study (Fritz et al., 1970; Pla and Fritz, 1971; Pla et al., 1973; Fritz et al., 1974) the method, as first conceived
by Pla and Fritz (1970), was refined and proposed to the Association of Official Analytical Chemists. The rat hemoglobin repletion test was subsequently adopted as the standard method of analysis for the bioavailability of iron by the Association of Official Analytical Chemists (AOAC, 1975, Sections 43.188-43.190). Since that time, changes recommended as a result of further collaborative study (Fritz et al., 1975; 1978) have been incorporated into the standard method (AOAC, 1980, Sections 43.217-43.219).

This procedure involves the feeding of a fast growing strain of male weanling rats a basal diet low in iron for a period of about 4 weeks. The rats are determined to be sufficiently anemic when hemoglobin levels are less than 6 g/dL. Once satisfactorily depleted the rats are divided into groups of ≥ 8 animals of similar mean body weight or hemoglobin level. Groups of test animals are placed on 0, 6, 12, and 24 mg Fe/kg diet, supplied by the reference standard FeSO$_4$·7H$_2$O. Other groups of animals are placed on diets that contain levels of test samples that provide similar concentrations of iron. The rats are individually housed in stainless steel cages and have free access to deionized distilled water throughout the repletion period. The diets are fed *ad libitum* for two weeks. At the end of the repletion period, tail blood is collected from each rat and hemoglobin levels are determined. Final hemoglobin values achieved by the iron test source are compared to those of the reference standard by the parallel lines technique of analysis (Bliss and White, 1967). The results are expressed as the relative biological value (RBV) of the test source relative to ferrous sulfate which is assigned a value of 100.
This technique has several advantages. The method employs an intact biological system, the method itself is relatively simple and available to a large number of researchers (Miller and Schricker, 1982). The experiments are of a relatively short duration (about one month) and a large number of iron sources can be screened fairly rapidly (Van Campen, 1983). The experimental animals are a relatively homogeneous population, and reproducibility among laboratories is generally good if identical or nearly identical conditions are used (Van Campen, 1983). Finally, this technique does take both the absorption and utilization of iron into account, although the relative contribution of the two processes cannot be separated (Van Campen, 1983).

There are also several disadvantages to the method. Formulation of the diet becomes a problem when this hemoglobin repletion technique is used to evaluate the intrinsic iron of whole foods (Fritz and Pla, 1972). The requirement for graded levels of iron in the diets means that when whole foods are used the composition of diets is usually not constant between groups (Miller and Schricker, 1982; Van Campen, 1983). However, the addition of whole foods to the basal diet is done in a manner to maintain protein and energy levels as nearly constant as possible between diets so that the diets have a similar effect on the growth of the animals (Fritz and Pla, 1972).

There is a difference of opinion among researchers as to the proper data analysis technique for the calculation of relative biological values of iron sources (Van Campen, 1983). Fritz et al. (1975) compared several statistical treatments (parallel lines, slope-ratio and graphic) for the
calculation of relative biological values of collaborative iron samples. It was observed that the different methods yielded similar conclusions regarding relative biological values of the iron sources. Fritz and co-workers postulated that if there were large significant differences in iron utilization between iron sources, these differences would be obvious regardless of the statistics applied to the data.

The hemoglobin repletion test probably overestimates the RBV of iron sources. The feeding of suboptimal levels of iron to depleted rats during a rapid stage of growth provides conditions whereby the animals can be expected to make the most efficient use of any iron given (Thompson and Raven, 1959). However, it is necessary to use depleted animals in order to measure a response to dietary iron (Miller and Schricker, 1982).

The results obtained by the hemoglobin repletion test are relative, rather than absolute, estimates of iron absorption. This limits the technique to ranking or screening a variety of iron sources (Van Campen, 1983).

Finally, there are many problems associated with the extrapolation of results from rats to humans (Miller and Schricker, 1982; Van Campen, 1983).

G. THE RAT AS A MODEL SYSTEM FOR PREDICTING IRON AVAILABILITY IN MAN

The rat is often used as an experimental model for predicting iron availability for humans. More often than not, physiological differences between the two species account for the different iron availability values obtained in the two species for the same iron sources.
Numerous studies have shown that ferrous iron is absorbed and utilized more efficiently by man than ferric iron (Narins, 1980), whereas rats absorb the ferrous and ferric forms equally well (Elwood, 1965).

The rat does appear to be a valid model for screening inorganic supplementary iron sources for man. The availabilities of selected inorganic iron sources to rats and man, as determined by animal repletion studies and human relative plasma iron responses following test sources of iron, were ranked in the same order by both species (ferrous sulfate > reduced iron > ferric orthophosphate and sodium iron pyrophosphate) (Pla and Fritz, 1971).

The rat is not a suitable model for evaluating the enhancing effect of ascorbic acid on nonheme dietary iron absorption. Fritz and Pla (1972) have postulated that species such as rats, which do not need a dietary source of ascorbic acid, produce enough ascorbic acid in their bodies to minimize any effect from dietary sources.

Among 21 food sources of iron tested in a collaborative study by the rat repletion technique (Fritz et al., 1970) no clear distinction in availability was observed between animal and vegetable foods. An extensive collaborative human study using the intrinsic radioiron labelling technique, determined that the iron of animal origin was more available to humans than that from vegetable sources (Martinez-Torres and Layrisse, 1974).

The absorption of hemoglobin iron by the rat, regardless of its iron status, is much lower than that demonstrated in normal (11%) and iron deficient human subjects (22%) (Callender et al., 1957; Turnbull et al.,
In studies using radioiron labelling techniques, Weintraub et al. (1965) found that in the normal adult rat, the absorption of a test dose of porphyrin iron (2%) was significantly less than that of a comparable dose of iron in the form of ferrous sulfate (11%). The comparably low level of absorption of hemin iron (1%) by the normal rat demonstrated that the poor absorption of hemoglobin iron was not due to the inability of the rat to split off the heme from the globin. It was observed that iron deficiency did not improve the absorption of hemoglobin iron (3%) while it had an effect on the absorption of ferrous sulfate iron (30%). That this inability to absorb heme iron was of physiological significance was demonstrated by producing a state of iron deficiency in animals raised on a diet with hemoglobin as the only source of iron. The results of their research led them to suggest that possibly the rat lacks the absorptive pathway for heme iron found in man and that perhaps there is a difference in the two species regarding the epithelial cells' ability to sequester iron as the salt or in the heme ring.
MATERIALS AND METHODS

A. ANIMAL CARE

Ninety male weanling Wistar rats, 35.7 ± 4.7 g in weight, were obtained from Woodlyn Laboratories Ltd., Guelph, Ont. The rats, prior to their arrival, had received only maternal milk; the shipping cages contained potato pieces but not stock diet. Upon arrival, they were housed singly in screen-bottomed stainless steel cages in facilities of the Animal Science Department, U.B.C. The temperature of the animal room was maintained at 23-25°C; the lighting was regulated automatically to provide alternate 12-hour periods of light and darkness (light on from 8:00 a.m. to 8:00 p.m.). Food and deionized distilled water were given ad libitum throughout the experimental period.

B. EXPERIMENTAL DIETS

1. General description

Nine experimental diets were used: a semipurified low-iron basal diet; the same basal diet to which was added 5, 10, 15, 20 or 25 mg iron per kg diet, supplied by the FeSO$_4$$\cdot$7H$_2$O reference standard (food grade FeSO$_4$$\cdot$7H$_2$O, Mallinckrodt, St. Louis, MO); and three test source diets which were formulated to provide a total of 15 mg iron per kg diet from either freeze-dried raw lean ground beef, a textured defatted soy flour meat extender or a 2.3:1 (w/w) combination of the freeze-dried lean ground beef and the textured defatted soy flour. Each of the nine diet treatments was assigned an alphabetic code as summarized in Table VI. The composition of the low-iron basal diet and that of the mineral and vitamin mixes are
shown in Table II while those of the diets containing test foods are outlined in Tables III, IV and V. All diets were formulated to supply the nutrient requirements of the growing rat (NRC, 1978), with the exception of iron; the reference sources for ingredient nutrient contents being Agriculture Handbook 8 (Watt and Merrill, 1963), scientific literature, supplier information and data obtained by chemical analysis. The diet ingredients were mixed by hand using a stainless steel bucket and culinary whisk. Efforts to preserve the nutritional and sensory properties of these diets included their being stored in the dark in sealed plastic bags at -22°C between experiments and 4°C during the feeding trials.

2. Preparation of test source ingredients
   
a. Beef

The total quantity of raw lean ground beef required for the preparation of beef-containing test diets was purchased at one time from a local retail outlet. The mean fat content of the beef, as determined by the Goldfisch ether extraction method on duplicate samples, was calculated to be 15.63% on a wet basis (63.33% moisture). The beef was pooled and mixed, weighed into 400 g lots, wrapped in pre-weighed sheets of aluminum foil and frozen overnight. The beef was freeze-dried (Thermovac Freeze Dryer, Model FDC-10, Thermovac Industries Corp., Marshalltown, IA), the average final moisture content achieved being 2.12%. The freeze-dried beef was then chopped in a Waring blender (Waring Products Division, Dynamics Corp. of America, New Hartford, CT) followed by hand grinding in porcelain mortar with a pestle. The resulting fibrous meal was stored in the dark at -22°C in sealed plastic bags until being incorporated into the test diets.
b. Textured defatted soy flour

A single sample of Textured Vegetable Protein (Unflavored, Fortified TVP®, Archer Daniels Midland Company, Decatur, IL), an extruded defatted soy flour product marketed in North America as a meat extender, was obtained through British Canadian Importers, Vancouver, B.C. The processing steps involved in the preparation of this soy product are outlined in Figure 1; the nutrient content of the retailed product is reported in Table I.

The soy product was physically converted to a form suitable for mixing into test diets by first chopping it in a Waring blender followed by hand grinding with a porcelain mortar and pestle. The product, in its final form, was a powder which passed through mesh No. 18 (1.00 mm diam). The ground soy product was stored at -22°C in sealed plastic bags until incorporation into test diets.

3. Formulation of test source diets

The three test diets containing food sources of iron were designed to be quantitatively similar with respect to iron contribution of the test sources as well as be alike in protein, fat and caloric content. Cellulose powder was added to adjust the diets accordingly. Overall, this group of diets was formulated to contain protein and energy levels comparable to that supplied by the basal diet.

The ratio of freeze-dried ground beef to unhydrated soy product (2.3:1, w/w) in the beef and soy flour diet was selected to approximate the formula recommended by the supplier for preparing a basic extended ground beef loaf: 10 lb extended beef = 7 lb raw ground beef + 1 lb meat extender
Figure 1. Processing scheme for TVP® Unflavored-Fortified product.¹,²,³

Whole Raw Soybeans

Heat

Crack, dehull, flake

Hypocotyl (3%) ("Germ")

Cotyledons (89%) (Full Fat Flakes)

Hulls (8%) ("Bran")

Solvent extract

Defatted Soy Flakes

Grind and classify

Defatted Soy Flour

Blend to a stiff slurry with water

Extrude

Dry and classify

Toast

Fortify with nutrients (Amino acids, vitamins, minerals)

Nutrient Fortified Textured Defatted Soy Flour Product

¹Archer Daniels Midland Company, Decatur, IL.
²Processing information obtained through British Canadian Importers, Vancouver, B.C.
³The percentage figures for the morphological components of the soybean seed are expressed on a wet weight basis (Circle and Smith, 1978).
Table I. Nutrient analysis for TVP® Unflavored-Fortified product.¹

<table>
<thead>
<tr>
<th>Nutrient</th>
<th>per 100 g</th>
<th>Vitamins</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water (g)</td>
<td>6.0</td>
<td></td>
</tr>
<tr>
<td>Total carbohydrate (g)</td>
<td>31.5</td>
<td>A</td>
</tr>
<tr>
<td>Fiber (g)</td>
<td>3.0</td>
<td>D</td>
</tr>
<tr>
<td>Total protein (g)</td>
<td>52.0</td>
<td>E</td>
</tr>
<tr>
<td>Amino acids</td>
<td></td>
<td>C</td>
</tr>
<tr>
<td>Tryptophan (mg)</td>
<td>343</td>
<td>Folacin (mg)</td>
</tr>
<tr>
<td>Threonine (mg)</td>
<td>2170</td>
<td>Thiamin (mg)</td>
</tr>
<tr>
<td>Isoleucine (mg)</td>
<td>2464</td>
<td>Riboflavin (mg)³</td>
</tr>
<tr>
<td>Leucine (mg)</td>
<td>4075</td>
<td>Niacin (mg)³</td>
</tr>
<tr>
<td>Lysine (mg)</td>
<td>3164</td>
<td>B6 (mg)³</td>
</tr>
<tr>
<td>Methionine (mg)</td>
<td>596</td>
<td>B12 (µg)³</td>
</tr>
<tr>
<td>Cystine (mg)</td>
<td>409</td>
<td>Biotin (µg)</td>
</tr>
<tr>
<td>Phenylalanine (mg)</td>
<td>2603</td>
<td>Pantothenate (mg)³</td>
</tr>
<tr>
<td>Tyrosine (mg)</td>
<td>1619</td>
<td>Minerals (g)</td>
</tr>
<tr>
<td>Valine (mg)</td>
<td>2617</td>
<td>Calcium (mg)</td>
</tr>
<tr>
<td>Arginine (mg)</td>
<td>3777</td>
<td>Phosphorus (mg)</td>
</tr>
<tr>
<td>Histidine (mg)</td>
<td>1347</td>
<td>Iron (mg)²³</td>
</tr>
<tr>
<td>Total fat (g)</td>
<td>1.0</td>
<td>Iodine (µg)</td>
</tr>
<tr>
<td>Calories</td>
<td>280.0</td>
<td>Chlorine (mg)</td>
</tr>
</tbody>
</table>

¹Archer Daniels Midland Company, Decatur, IL.

²The endogenous iron content of the soy flour (8 mg/100 g, wb) is supplemented with ferrous sulfate to bring the total iron content of the product to 10 mg/100 g, wb.

³Nutrients added in excess of levels reported for TVP® Unflavored, Non-fortified product.
Table II. Composition of basal diet.¹

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Skim milk powder</td>
<td>53.00</td>
</tr>
<tr>
<td>Glucose</td>
<td>35.96</td>
</tr>
<tr>
<td>Corn oil</td>
<td>5.00</td>
</tr>
<tr>
<td>Cellulose powder</td>
<td>2.70</td>
</tr>
<tr>
<td>Potassium chloride</td>
<td>2.05</td>
</tr>
<tr>
<td>Vitamin mix²</td>
<td>1.00</td>
</tr>
<tr>
<td>Choline chloride</td>
<td>0.20</td>
</tr>
<tr>
<td>Potassium phosphate monobasic</td>
<td>0.07</td>
</tr>
<tr>
<td>Mineral mix⁶</td>
<td>0.02</td>
</tr>
</tbody>
</table>

¹4.2 ppm total iron (db).
²Lucerne brand (Canada Safeway Ltd., Winnipeg, Man.).
³Mazola brand (Canada Starch Co., Inc., Montreal, P.Q.).
⁴Alphacel (ICN Nutritional Biochemicals, Cleveland, OH).
⁵AIN vitamin mixture 76 (American Institute of Nutrition, 1977) (10663 - U.S. Biochemical Corp., Cleveland, OH) supplying per kg diet: 6.0 mg thiamin HCl, 6.0 mg riboflavin, 30.0 mg nicotinic acid, 7.0 mg pyridoxine HCl, 2.0 mg folic acid, 10.0 μg cyanocobalamin, 16.0 mg D-calcium pantothenate, 0.2 mg D-biotin, 4,000 I.U. vitamin A, 1,000 I.U. vitamin D₃, 50 I.U. vitamin E and 50 μg vitamin K.
⁶Mineral mix supplying per kg diet: 0.85 mg K₂Cr₂O₇, 20.0 mg CuSO₄•5H₂O, 2.25 mg NaF, 0.20 mg KI, 154.0 mg MnSO₄•H₂O, 0.22 mg Na₂SeO₃ and 15.0 mg ZnO.
Table III. Composition of beef diet.

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Skim milk powder</td>
<td>29.50</td>
</tr>
<tr>
<td>Cellulose powder</td>
<td>23.00</td>
</tr>
<tr>
<td>Glucose</td>
<td>21.99</td>
</tr>
<tr>
<td>Freeze-dried raw ground beef(^1)</td>
<td>20.01</td>
</tr>
<tr>
<td>Corn oil</td>
<td>3.00</td>
</tr>
<tr>
<td>Potassium chloride</td>
<td>0.92</td>
</tr>
<tr>
<td>Vitamin mix(^2)</td>
<td>1.00</td>
</tr>
<tr>
<td>Choline chloride</td>
<td>0.20</td>
</tr>
<tr>
<td>Calcium carbonate</td>
<td>0.28</td>
</tr>
<tr>
<td>Potassium phosphate monobasic</td>
<td>0.07</td>
</tr>
<tr>
<td>Mineral mix(^2)</td>
<td>0.02</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>0.01</td>
</tr>
</tbody>
</table>

\(^1\)Moisture content of 2.12%.

\(^2\)As for basal diet (Table II).
### Table IV. Composition of beef and soy flour diet.

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Skim milk powder</td>
<td>30.78</td>
</tr>
<tr>
<td>Glucose</td>
<td>21.29</td>
</tr>
<tr>
<td>Cellulose powder</td>
<td>19.79</td>
</tr>
<tr>
<td>Freeze-dried raw ground beef&lt;sup&gt;1&lt;/sup&gt;</td>
<td>12.59</td>
</tr>
<tr>
<td>Corn oil</td>
<td>7.30</td>
</tr>
<tr>
<td>Textured defatted soy flour&lt;sup&gt;2&lt;/sup&gt;</td>
<td>5.55</td>
</tr>
<tr>
<td>Potassium chloride</td>
<td>1.18</td>
</tr>
<tr>
<td>Vitamin mix&lt;sup&gt;3&lt;/sup&gt;</td>
<td>1.00</td>
</tr>
<tr>
<td>Choline chloride</td>
<td>0.20</td>
</tr>
<tr>
<td>Calcium carbonate</td>
<td>0.25</td>
</tr>
<tr>
<td>Potassium phosphate monobasic</td>
<td>0.05</td>
</tr>
<tr>
<td>Mineral mix&lt;sup&gt;3&lt;/sup&gt;</td>
<td>0.02</td>
</tr>
</tbody>
</table>

<sup>1</sup>Moisture content of 2.12%.

<sup>2</sup>Ground, Unflavored, Fortified TVP<sup>®</sup> (Archer Daniels Midland Company, Decatur, IL).

<sup>3</sup>As for basal diet (Table II).
Table V. Composition of soy flour diet.

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Skim milk powder</td>
<td>32.97</td>
</tr>
<tr>
<td>Glucose</td>
<td>19.98</td>
</tr>
<tr>
<td>Textured defatted soy flour(^1)</td>
<td>14.99</td>
</tr>
<tr>
<td>Corn oil</td>
<td>14.59</td>
</tr>
<tr>
<td>Cellulose powder</td>
<td>13.99</td>
</tr>
<tr>
<td>Potassium chloride</td>
<td>2.11</td>
</tr>
<tr>
<td>Vitamin mix(^2)</td>
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</tr>
<tr>
<td>Choline chloride</td>
<td>0.20</td>
</tr>
<tr>
<td>Calcium carbonate</td>
<td>0.15</td>
</tr>
<tr>
<td>Mineral mix(^2)</td>
<td>0.02</td>
</tr>
</tbody>
</table>

\(^1\)Ground, Unflavored, Fortified TVP® (Archer Daniels Midland Company, Decatur, IL).

\(^2\)As for basal diet (Table II).
Table VI. Repletion phase diet treatments.

<table>
<thead>
<tr>
<th>Treatment code</th>
<th>Diet treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Basal diet&lt;sup&gt;1&lt;/sup&gt;</td>
</tr>
<tr>
<td>B</td>
<td>Basal diet + 5 mg iron (as FeSO₄·7H₂O)/kg diet</td>
</tr>
<tr>
<td>C</td>
<td>Basal diet + 10 mg iron (as FeSO₄·7H₂O)/kg diet</td>
</tr>
<tr>
<td>D</td>
<td>Basal diet + 15 mg iron (as FeSO₄·7H₂O)/kg diet</td>
</tr>
<tr>
<td>E</td>
<td>Basal diet + 20 mg iron (as FeSO₄·7H₂O)/kg diet</td>
</tr>
<tr>
<td>F</td>
<td>Basal diet + 25 mg iron (as FeSO₄·7H₂O)/kg diet</td>
</tr>
<tr>
<td>G</td>
<td>Freeze-dried ground beef</td>
</tr>
<tr>
<td>H</td>
<td>Freeze-dried ground beef + textured defatted soy flour (2.3:1, w/w)</td>
</tr>
<tr>
<td>I</td>
<td>Textured defatted soy flour</td>
</tr>
</tbody>
</table>

<sup>1</sup>Diet also used in depletion phase to make rats anemic. In repletion phase the diet served as a negative control.
+ 2 lb water, while at the same time maintaining the total iron contribution by these ingredients at 15 ppm.

C. BIOLOGICAL ASSAY

The availability of the iron contained in the test diets was determined using the hemoglobin repletion procedure described by Pla and Fritz (1970) and later adopted by the AOAC as official first action (AOAC, 1980, Sections 43.217-43.219). The rats were fed the low-iron basal diet until they were satisfactorily depleted of their iron stores i.e. hemoglobin < 6 g/dL, hematocrit < 29% (Pla and Fritz, 1970). Two weeks after the rats were put on the basal diet their hematological indices were measured. Blood samples were taken by tailcutting after anesthetizing the rats with anhydrous ether (for anesthesia) (J. T. Baker Chemical Co., Phillipsburg, NJ). Blood for hemoglobin determinations was directly drawn from the tail vein into oxalate coated calibrated sampling pipets (Corning Glass Works, Corning, NY). Similarly, blood samples for hematocrit measurement were drawn into even bore heparinized micro-hematocrit capillary tubes (Fisher Scientific Co., Fair Lawn, NJ) to the 3/4 mark; the tubes were sealed at one end with clay. Only single blood samples were taken for each test per sampling session due to the rapid onset of blood clotting within the tail vein.

After two weeks it was found that the rats were not satisfactorily depleted so they were continued on the basal diet for an additional week. At the end of the third week measurements of the blood parameters were repeated and accepted as being sufficiently low to terminate the depletion phase of the experiment. At the end of 21 days of depletion, the mean
values (± standard deviation) for hemoglobin and hematocrit were 3.47 ± 0.73 g/dL and 14.69 ± 2.25%, respectively (Appendix B and C). During the depletion phase individual rat weights and food consumptions were recorded weekly.

The rats were started on the repletion phase the same day they were assessed to be sufficiently iron deplete. The rats were grouped according to similarity in weight (Appendix B and C); in turn, these groups were randomly assigned to one of the nine experimental diets described earlier (Table VI). Food and deionized distilled water were provided ad libitum throughout the repletion phase. As only 30 rats were ordered at a time, the depletion/repletion procedure was conducted on three occasions so that for the nine diets tested there were 10 observations for each type of response measured. After two weeks on the experimental diets, individual blood samples were taken for hemoglobin and hematocrit determinations. Individual rat weights and food consumptions were recorded weekly.

D. HEMOGLOBIN AND HEMATOCRIT DETERMINATION

Hemoglobin was determined by the cyanmethaemoglobin method of Crosby et al. (1954). 0.02 mL of blood was diluted with 5 mL of Drabkin's reagent and the absorbance of the solution was read at 540 nm using a Beckman double beam spectrophotometer (Beckman Industries, Fullerton, CA) which had been set at zero absorbance with Drabkin's reagent. The concentration of cyanmethemoglobin pigment was calculated from a standard curve prepared with cyanmethemoglobin standards (Laboratory Centre for Disease Control, Department of National Health and Welfare, Ottawa, Ont.) made up in Drabkin's reagent. The results were expressed as g hemoglobin/dL.
Hematocrit values were determined according to the method of Cohen (1967). Blood-filled hematocrit tubes were centrifuged (International Micro-capillary Centrifuge, Model MB, International Equipment Co., Needham Heights, MA) at 12,000 rpm for 6 min. Hematocrit (% packed red cell volume) was calculated by measuring with a 150 mm ruler the height of the packed red cells and dividing this value by the total height of red cells and plasma.

E. CALCULATION OF IRON BIOAVAILABILITY

The hematological responses of rats fed the basal diet containing graded levels of ferrous sulfate reference standard were plotted as a standard curve and the equation of the line determined by linear regression analysis using an Apple II personal computer (Apple Computer, Inc., Cupertino, CA) and curve fitting program package (Warme, 1980). The responses of rats fed test sources of iron were related to the standard curve by comparing the iron levels required to achieve the same hematological response. The relative biological value (RBV) of the test source was calculated according to the following equation

\[
\text{RBV test source} = 100 \times \frac{\text{mgFe/kg from FeSO}_4 \cdot 7\text{H}_2\text{O}}{\text{mgFe/kg from test source}} \text{; giving the same response in hemoglobin (or hematocrit) (Pla and Fritz, 1971).}
\]

F. PROXIMATE ANALYSIS OF DIET INGREDIENTS AND DIETS

Proximate analysis was performed on the iron test source ingredients, the low-iron basal diet and the three test source diets. The defatted soy protein product and diets containing the soy product were analyzed for
phytic acid content. Basal diet containing graded levels of ferrous sulfate was analyzed for moisture and total iron content only. Chemical analysis of the cellulose powder included quantitative determination of moisture, neutral detergent fiber, total iron and magnetically extractable iron.

1. Moisture

The AOAC vacuum oven method for wheat flour (AOAC, 1980, Section 14.002), with some modification, was used to determine the moisture content of the diets and individual ingredients. Triplicate samples (2 g) were accurately weighed into pre-dried, desiccator-cooled, weighed aluminum dishes (50.8 mm diam x 22.4 mm depth) with tight fitting slip-over covers (Canlab, Richmond, B.C.). Samples were dried to a constant weight in a vacuum oven (National Appliances Co., Portland, OR) under 91.5 kPA (27 inches Hg) at 100°C for defatted soy flour and cellulose powder samples and 80°C for diet and freeze-dried beef samples. The loss in sample weight was recorded as moisture.

2. Crude protein

Protein content was determined by the macro-Kjeldahl technique of the AOAC (AOAC, 1980, Section 2.057) with the following modifications. Triplicate 1 g samples were digested for 1 h in 35 mL H₂SO₄ (conc) plus added catalyst (10.4 g of a K₂SO₄-CuSO₄ - pumice mixture, 10:3:1 w/w/w). Distillates were titrated with HCl (0.1091 N) previously standardized with THAM (tris hydroxy methylamino methane) (Fisher Scientific Co., Fair Lawn, NJ); the number of mLs HCl per titration being recorded.
% N (db) of each sample was calculated by the following equation

\[
\frac{N \text{ HCl} \times 14.007 \times \text{mL HCl used}}{\text{g dry weight of sample} \times 1000} \times 100
\]

Values for crude protein content (% db) were obtained by multiplying the % N (db) by the following nitrogen to protein conversion factors: 6.38 for the milk-based basal diet and 6.25 for the beef or beef and soy-based diets.

3. Ether extract

Crude fat determinations were performed in triplicate using a Goldfisch fat extraction unit (Labconco Corp., Kansas City, MO). Prior to extraction, all samples were vacuum oven dried to a constant weight and held in a desiccator containing silica gel. Similarly, the beakers used in the extraction procedure were dried, weighed and desiccated until the time of extraction. 1 g dried samples were transferred to Whatman cellulose extraction thimbles (22 mm diam x 80 mm ht) (VWR Scientific Inc., Brisbane, CA) containing a small amount of glass wool packing at the tip. The samples were extracted for 4 h on the low heat setting of the unit with 35 mL petroleum ether. The extraction beakers were removed from the unit, placed in a fume hood to volatilize the remaining ether (6 h), oven dried (85°C) overnight (8 h), desiccator cooled (1 h) and weighed. The crude fat contents of the beakers were calculated and reported as % ether extract (db).

4. Neutral detergent fiber

The neutral detergent fiber content of samples was analyzed in trip-
licate by the micro-digestion procedure developed by Waldern (1971) employing the neutral-detergent solution of Van Soest and Wine (1967). Sample preparation included extraction for 6 h with petroleum ether using Soxhlet extraction apparatus (Fisher Scientific Co., Fair Lawn, NJ) followed by overnight oven drying at 50°C to volatilize the solvent. Particulate matter remaining after the filtration of digested sample was vacuum oven dried overnight (12 h) at 100°C under 91.5 kPa, cooled in a desiccator over silica gel and weighed. Sample fiber contents were calculated from the recorded dry weights employing a correction for crude fat content and reported as % neutral detergent fiber (db).

5. Ash

The ash content of samples was determined in triplicate using the AOAC dry ashing technique for wheat flour (AOAC, 1980, Section 14.006) with the following modification. The samples were carbonized on a hot plate prior to their being ignited in the muffle furnace. Sample ash contents were calculated from recorded ash weights and reported as % ash (db).

6. Iron

a. Total iron

Total iron concentration of samples was determined in triplicate on wet ashed samples by atomic absorption spectrophotometry. All glassware used during the analytical procedure was washed, soaked overnight in 1 N HCl and rinsed with deionized distilled water. All reagent solutions used in total iron determination were prepared with deionized distilled water.
The wet ashing procedure involved the digesting of 1.2 g samples with 15 mL of a mixture of 25% hydrochloric and 65% nitric acids (9:1, v/v) and 10 mL of water for 1/2 h over a hot water bath (80-85°C) as described by Maurer (1977). The digested sample was cooled, made up to volume (50 mL) with water and filtered through ashless filter paper (Whatman 541, VWR Scientific Inc., Brisbane, CA). The iron concentration of the clarified solution was measured at 248.3 nm by a double beam atomic absorption spectrophotometer (Perkin Elmer Corp., Norwalk, CT, Model 306) using an iron-specific hollow cathode lamp, 5 cm single-slot burner head and air-acetylene flame. The instrument was calibrated with iron standards prepared from Certified Iron Reference Solution (Fisher Scientific Co., Fair Lawn, NJ) diluted with HCl-HNO₃ acid mixture and deionized distilled water. A reagent blank was carried through the entire ashing procedure for every five samples analyzed. Sample readings obtained by atomic absorption spectrophotometry were corrected for their respective reagent blank readings before calculating the iron concentration of samples on a dry weight basis.

b. Magnetically extractable iron

Total iron analysis of the cellulose powder indicated this diet ingredient to contain a high level of iron. Further analysis was performed in order to determine the derivation of this iron.

The magnetically extractable iron content was determined on duplicate samples of cellulose powder according to the method described by Lee and Clydesdale (1979b). The teflon coated magnets bearing the iron extracted from the sample slurry (5 g sample blended with 70 mL deionized distilled
water) were soaked in concentrated HCl until the iron had dissolved from
the magnets. The HCl-iron solution was made up to volume with deionized
distilled water and the iron concentration measured by atomic absorption
spectrophotometry as described in Section 6a. The instrument was cali-
brated with iron standards prepared from Certified Iron Reference Solution
(Fisher Scientific Co., Fair Lawn, NJ) diluted with 20% HCl. The concen-
tration of iron in the sample slurry was calculated on a dry weight basis.

7. Phytic acid

The method of Latta and Eskin (1980) was used to determine the phytic
acid contents of the defatted soy flour product and diet samples containing
the soy product. Aliquots of sample extract were eluted through chloride
anion-exchange resin. Inorganic phosphorus was eluted with 0.1 M NaCl
followed by elution of phytate with 0.7 M NaCl. The absorbance of a solu-
tion of phytate extract and Wade reagent (3:1, v/v) was read at 500 nm
using a Cary 210 spectrophotometer (Varian Associates Inc., Palo Alto, CA)
which had been set at zero absorbance with deionized distilled water. The
concentration of phytic acid was calculated from a standard curve prepared
with sodium phytate standards (inositol hexaphosphoric acid, corn type V,
97% pure, sodium salt) (Sigma Chemical Co., St. Louis, MO) made up in
deionized distilled water and reacted with Wade reagent. The procedure for
phytic acid determination was performed in triplicate for each sample and
the calculated sample concentrations reported on a dry weight basis.
G. STATISTICAL ANALYSIS

The raw data collected for the biological assay was analyzed in a one-way analysis of variance, using the UBC MFAV program package (Le, 1978) available for use on the UBC Amdahl 470 V/8 computer. Duncan's multiple range test (Zar, 1974) was used to perform multiple comparisons among means.
RESULTS AND DISCUSSION

A. COMPOSITION OF THE EXPERIMENTAL DIETS

The composition of the test foods and fiber source used in formulating the diets and that of the 9 experimental diets are presented in Tables VII and VIII, respectively. Values experimentally determined are compared to reported values in Appendix A.

The results of analysis indicate the overall design objective for the 9 experimental diets, that being maintaining the protein and energy levels constant between diets, was achieved. It is well established that these nutritional factors influence growth. At suboptimal levels of dietary iron the degree of anemia is correlated with the growth of the young animal (Fritz and Pla, 1972).

The group of diets containing iron from test sources were designed to supply on a per kg basis similar levels of protein, fat, calories and iron (15 ppm). The level of protein and calories were kept constant for growth purposes; the levels of fat in each diet was held constant in an effort to maintain the same satiating effect between diets. It has been hypothesized that the emptying of food into the intestine is necessary for feeding to stop and for the satiety sequence to appear (Liebling et al., 1974). It is well established that the presence of fat in the diet delays gastric emptying. Therefore, it would appear reasonable to assume that a constant level of fat between diets would be expected to reduce differences in feeding behavior. The decision to select 15 ppm as the iron test dose was based on the results of rat repletion assays performed on graded levels of ferrous sulfate (10, 20 and 40 ppm iron) by Amine et al. (1972). In this
Table VIII. Proximate composition of basal, beef, beef and soy flour, and soy flour diets.

<table>
<thead>
<tr>
<th>Diet Treatment</th>
<th>Dry Matter (%)</th>
<th>Energy (kcal/g)</th>
<th>Crude Protein (%)</th>
<th>Ether Extract (%)</th>
<th>Detergent Fiber (%)</th>
<th>Ash (%)</th>
<th>Total Iron (ppm)</th>
<th>Phytic Acid (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal</td>
<td>95.89&lt;sup&gt;1,2&lt;/sup&gt;</td>
<td>3.85</td>
<td>18.50</td>
<td>4.82</td>
<td>2.54</td>
<td>6.71</td>
<td>4.20&lt;sup&gt;4&lt;/sup&gt;</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>(0.23)</td>
<td>(0.22)</td>
<td>(0.21)</td>
<td>(0.09)</td>
<td>(0.09)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Beef</td>
<td>96.66</td>
<td>3.56</td>
<td>19.25</td>
<td>11.29</td>
<td>21.80</td>
<td>4.45</td>
<td>27.80</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>(0.34)</td>
<td>(0.53)</td>
<td>(0.09)</td>
<td>(0.41)</td>
<td>(0.05)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Beef + soy flour (2.3:1, w/w)</td>
<td>96.77</td>
<td>3.64</td>
<td>19.25</td>
<td>12.52</td>
<td>19.95</td>
<td>5.07</td>
<td>28.55</td>
<td>0.11</td>
</tr>
<tr>
<td></td>
<td>(0.19)</td>
<td>(0.13)</td>
<td>(0.05)</td>
<td>(0.13)</td>
<td>(0.01)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Soy flour</td>
<td>96.92</td>
<td>3.78</td>
<td>19.72</td>
<td>13.65</td>
<td>12.92</td>
<td>6.27</td>
<td>30.63</td>
<td>0.33</td>
</tr>
<tr>
<td></td>
<td>(0.14)</td>
<td>(0.58)</td>
<td>(0.31)</td>
<td>(0.62)</td>
<td>(0.05)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>1</sup>Mean ± (standard deviation) of 3 determinations.

<sup>2</sup>Expressed on dry weight basis.

<sup>3</sup>Calculated on basis of data values in Watt and Merrill (1963).

<sup>4</sup>The total iron content of the basal diet to which was added 5, 10, 15, 20 or 25 mg iron (as FeSO₄·7H₂O)/kg was 9.3, 14.1, 18.4, 23.7 and 28.4 ppm (db), respectively.
Table VIII. Proximate composition of basal, beef, beef and soy flour, and soy flour diets.

<table>
<thead>
<tr>
<th>Diet Treatment</th>
<th>Dry Matter (%)</th>
<th>Energy[^3] (kcal/g)</th>
<th>Crude Protein (%)</th>
<th>Ether Extract (%)</th>
<th>Neutral Detergent Fiber (%)</th>
<th>Ash (%)</th>
<th>Total Iron (ppm)</th>
<th>Phytic Acid (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal</td>
<td>95.89[^1,2]</td>
<td>3.85</td>
<td>18.50</td>
<td>4.82</td>
<td>2.54</td>
<td>6.71</td>
<td>4.20[^4]</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>(0.23)</td>
<td></td>
<td>(0.22)</td>
<td>(0.21)</td>
<td>(0.09)</td>
<td>(0.09)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Beef</td>
<td>96.66</td>
<td>3.56</td>
<td>19.25</td>
<td>11.29</td>
<td>21.80</td>
<td>4.45</td>
<td>27.80</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>(0.34)</td>
<td></td>
<td>(0.53)</td>
<td>(0.09)</td>
<td>(0.41)</td>
<td>(0.05)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Beef + soy flour (2.3:1,w/w)</td>
<td>96.77</td>
<td>3.64</td>
<td>19.25</td>
<td>12.52</td>
<td>19.95</td>
<td>5.07</td>
<td>28.55</td>
<td>0.11</td>
</tr>
<tr>
<td></td>
<td>(0.19)</td>
<td></td>
<td>(0.13)</td>
<td>(0.05)</td>
<td>(0.13)</td>
<td>(0.01)</td>
<td>(0.02)</td>
<td>(0.02)</td>
</tr>
<tr>
<td>Soy flour</td>
<td>96.92</td>
<td>3.78</td>
<td>19.72</td>
<td>13.65</td>
<td>12.92</td>
<td>6.27</td>
<td>30.63</td>
<td>0.33</td>
</tr>
<tr>
<td></td>
<td>(0.14)</td>
<td></td>
<td>(0.58)</td>
<td>(0.31)</td>
<td>(0.62)</td>
<td>(0.05)</td>
<td>(2.18)</td>
<td>(0.01)</td>
</tr>
</tbody>
</table>


[^4]The total iron content of the basal diet to which was added 5, 10, 15, 20 or 25 mg iron (as FeSO₄·7H₂O)/kg was 9.3, 14.1, 18.4, 23.7 and 28.4 ppm (db), respectively.
study it was found that the dose-response line remained linear up to a level of 20 ppm iron but departed from linearity thereafter. As well, the hemoglobin concentrations of animals repleted with ferrous sulfate up to the level of 20 ppm were below that considered normal for the rat, while those of rats supplied with 40 ppm iron had reached normal levels by the end of the third week of repletion. An iron test dose of 15 ppm was therefore selected on the assumption that the ferrous sulfate reference dose-response curve would be linear in this region of the curve and the rats would still be engaged in the process of hemoglobin regeneration.

The total iron content of these diets, while similar in level, ranging from 27.8 to 30.6 ppm (db), was about twice that originally intended. This was due to the iron contributed by the fiber source. As Table VII shows, the total iron content of the cellulose powder was approximately 77 ppm (db) of which 18 ppm (db) was magnetically extractable and assumed to originate from the metal surface of the mill used to reduce the cellulose to a fine powder. The addition of a non-nutritive fiber source, at levels ranging from 150 to 230 g per kg of diet formulation, was necessary in order to maintain protein, fat and caloric levels constant between the diets.

**B. FEED INTAKE AND GROWTH**

The feed intake and growth for rats fed diets containing food test sources are reported in Table IX while those for rats repleted by graded levels of ferrous sulfate appear in Appendix C.

The mean feed intake of rats fed either of the beef-containing diets was greater than that of animals fed the ferrous sulfate supplemented basal
Table IX. Feed intake and body weight gain for iron depleted rats fed test sources of iron during the repletion period.

<table>
<thead>
<tr>
<th>Diet treatment</th>
<th>Feed intake (g)³</th>
<th>Weight gain (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal + 25 mg iron (as FeSO₄·7H₂O)/kg</td>
<td>181.8ab¹,² (30.5)</td>
<td>77.6a (16.3)</td>
</tr>
<tr>
<td>Beef</td>
<td>216.1c (16.4)</td>
<td>/1.4a (12.7)</td>
</tr>
<tr>
<td>Beef + soy flour (2.3:1, w/w)</td>
<td>199.6bc (20.4)</td>
<td>74.8a (16.2)</td>
</tr>
<tr>
<td>Soy flour</td>
<td>176.1a (18.3)</td>
<td>77.0a (15.9)</td>
</tr>
</tbody>
</table>

¹Mean of 10 animals ± (standard deviation).

²Means in a column followed by the same letter are not significantly different (P > 0.05) as determined by Duncan's multiple range test.

³Expressed on dry weight basis.
diet or the diet containing soy flour. However, this higher level of consumption of diets containing beef was not reflected in the weight gain of animals fed these diets. The lower caloric density of the beef-containing diets (Table VIII) did not account for the increased consumption. The differences in mean feed intake for the three test source diets appear to correlate with the crude fat content of these diets (Table VIII), however, no definite conclusion can be made regarding the effect of fat level on animal feeding behavior.

A comparison of the mean weight gains for all 9 diets showed there to be no significant differences (P > 0.05) in body weight gain by rats fed the basal diet supplemented with 10, 15, 20 and 25 ppm iron as ferrous sulfate or the three test source diets. Furthermore, the weight gains for these diets were significantly greater (P < 0.05) than those for rats fed either the basal diet without ferrous sulfate or the basal diet supplemented with 5 ppm iron as ferrous sulfate. The greater gain in weight observed in the animals fed higher levels of iron could be interpreted as an improved health status resulting from a faster rate of iron repletion (Mahoney and Hendricks, 1976). The weight gain by rats supplemented with ferrous sulfate compared favorably to experimental values obtained in studies using similar methodology (Rotruck and Luhrsen, 1979; Shah et al., 1983).

C. HEMOGLOBIN AND HEMATOCRIT RESPONSES

The final hemoglobin levels and hematocrit values for rats fed iron as food test sources or as ferrous sulfate are reported in Table X and Appendix C, respectively.
Table X. Final hemoglobin levels and hematocrit values for iron depleted rats fed test sources of iron during the repletion period.

<table>
<thead>
<tr>
<th>Diet treatment</th>
<th>Final hemoglobin (g/dL)</th>
<th>Final hematocrit (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal + 25 mg iron (as FeSO₄·7H₂O)/kg</td>
<td>10.1b₁,₂ (1.8)</td>
<td>35.5bc (4.6)</td>
</tr>
<tr>
<td>Beef</td>
<td>7.7a (0.9)</td>
<td>30.6a (2.5)</td>
</tr>
<tr>
<td>Beef + soy flour (2.3:1, w/w)</td>
<td>8.7a (0.7)</td>
<td>32.7ab (2.5)</td>
</tr>
<tr>
<td>Soy flour</td>
<td>10.8b (1.3)</td>
<td>37.8c (4.4)</td>
</tr>
</tbody>
</table>

¹Mean of 10 animals ± (standard deviation).

²Means in a column followed by the same letter are not significantly different (P > 0.05) as determined by Duncan's multiple range test.
The hemoglobin levels and hematocrit values for rats fed the basal diet supplemented with graded levels of ferrous sulfate reference standard increased progressively as the iron dose became higher, the mean hematological response at each iron level being significantly higher \( (P < 0.05) \) than the response at the dose below. The dose-response relationship observed in this study fulfills what is considered to be an appropriate criterion of a good bioavailability assay (Mahoney and Hendricks 1982). The hematological responses of rats fed the highest level of iron as added ferrous sulfate (25 ppm, db) were below the reported normal range of values for the rat (12-17.5 g hemoglobin/dL blood, 39-53% hematocrit) (Spector, 1956), thus indicating that at this level of iron the rats were still actively involved in the regeneration of hemoglobin and erythrocytes. This suboptimal hematological response by the rats is also regarded as a criterion of a good assay.

At the end of two weeks of repletion the mean hemoglobin concentration of rats fed the soy flour diet was not significantly different \( (P > 0.05) \) from that of rats fed the basal diet supplemented with 25 ppm iron as ferrous sulfate; the final hemoglobin concentrations of rats consuming these two diets were significantly higher \( (P < 0.05) \) than those of rats fed either the beef diet or beef and soy flour diet.

There was considerable overlap of mean response values for rats repleted with the test source diets and the ferrous sulfate supplemented basal diet when the final hematocrit value was used as the criterion of response to the iron source. The hematocrit values for the soy flour diet was significantly greater \( (P < 0.05) \) than those for the beef diet and beef
and soy flour diet; the response by rats fed the supplemented basal diet was significantly greater (P < 0.05) than that by rats repleted with the beef diet.

D. REGRESSION ANALYSIS

Standard hematological response curves for the iron reference salt (ferrous sulfate) were derived from the hemoglobin concentrations and hematocrit values determined for blood samples taken from rats repleted for two weeks with the low-iron basal diet and basal diet supplemented with graded levels of ferrous sulfate. The response data were plotted against the iron concentrations of the 6 reference diets, corrected for the iron contributed by the basal diet ingredients (Figure 2), using the linear least squares curve fitting technique. Visual inspection of the data points in relation to the regression line obtained suggested that the curve was indeed linear up to 15 ppm added iron, but beyond this iron level there was a downward trend in the fit of data points to the curve. The slopes of individually calculated dose-response regression lines (final hemoglobin) in the range of 0 to 15, 0 to 20 and 0 to 25 ppm added iron were respectively 0.391, 0.341 and 0.324, thus confirming curvature to be present. However, despite the curvature, responses to even the highest level of iron had to be included in the curve in order to accommodate the calculation of RBVs of iron in the test source diets. Thus, the curves from which the RBVs were calculated were found to be well described by the following regression equations:

\[
\text{Final hemoglobin} \quad Y = 2.673 + 0.324X \quad r^2 = 0.8328 \quad s_{Y\cdot X} = 1.215
\]

\[
\text{Final hematocrit} \quad Y = 12.775 + 1.034X \quad r^2 = 0.8292 \quad s_{Y\cdot X} = 3.862
\]
Figure 2. Two week hemoglobin response of iron depleted rats to graded levels of ferrous sulfate reference standard.

\[ Y = 2.673 + 0.324X \]

\[ r^2 = 0.8328 \]
The standard response curve for hemoglobin is presented in Figure 2. The distribution of data points about the regression line is representative of that obtained for the hematocrit response curve as well.

Alternatively, the hemoglobin response data for all levels of iron were plotted by the polynomial least squares curve fitting technique. The $r^2$ value obtained by this plotting technique was 0.8467.

E. RELATIVE BIOLOGICAL VALUE OF IRON IN TEST SOURCE DIETS

The relative bioavailability of the iron in the beef, beef and soy flour, and soy flour diets are reported in Table XI. The RBVs were calculated using the total iron values for the test source diets as determined by chemical analysis (Table VIII). Had the total iron values for the test source diets been corrected for iron contributed by the cellulose powder, then the RBVs calculated on the basis of either hematological response for all food test sources would have greatly exceeded that demonstrated for ferrous sulfate, which would have been in contradiction to the literature reported for the rat. As the majority of animal researchers, in evaluating the relative iron availability in beef, soy and beef-soy mixtures, employed hemoglobin repletion methodology, all RBVs referred to from the present study are those calculated from final hemoglobin responses.

1. Beef

The RBV of iron in freeze-dried raw ground beef (56%) compared favorably to the value of 53% reported by others (Shah et al., 1983) for
Table XI. Relative biological value (RBV) of iron in iron test source diets (FeSO₄·7H₂O = 100.0).

<table>
<thead>
<tr>
<th>Diet treatment</th>
<th>RBV³</th>
<th>Final hematocrit</th>
<th>95% confidence limits</th>
<th>RBV³</th>
<th>Final hemoglobin</th>
<th>95% confidence limits</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beef</td>
<td>55.7a¹</td>
<td>48.6 - 62.8</td>
<td></td>
<td>61.9a</td>
<td>55.3 - 68.5</td>
<td></td>
</tr>
<tr>
<td>Beef + soy flour (2.3:1, w/w)</td>
<td>65.3b</td>
<td>59.7 - 71.0</td>
<td></td>
<td>67.6a</td>
<td>61.2 - 74.0</td>
<td></td>
</tr>
<tr>
<td>Soy flour</td>
<td>81.3c</td>
<td>71.7 - 90.9</td>
<td></td>
<td>79.0b</td>
<td>68.5 - 89.5</td>
<td></td>
</tr>
</tbody>
</table>

¹Mean of 10 animals.

²Means in a column followed by the same letter are not significantly different (P > 0.05) as determined by Duncan's multiple range test.

³Relative biological value = 100 x (mgFe/kg from FeSO₄·7H₂O)/(mg Fe/kg from test source) giving the same hematological response.
anemic rats. The somewhat lower RBV of 41% reported by Rotruck and Luhrsen (1979) may possibly have been due to cooking of beef before feeding.

The low availability of beef iron to rats in the present study was not unexpected. Weintraub et al. (1965) demonstrated that the rat, regardless of its iron status, does not absorb porphyrin iron as well as it does iron in the inorganic form as ferrous sulfate.

In human studies the absorption of intrinsically labelled iron of veal muscle was not significantly different from that of a tracer dose of iron as ferric chloride or iron ascorbate (Layrisse and Martinez-Torres, 1972). Thus, the relative availability of iron in veal muscle, which is similar to that in ground beef, was 100% of the standard, almost twice that observed in the present study with rats.

2. Soy protein

The RBV of iron in the ferrous sulfate fortified textured defatted soy product (81%) falls within the broad range of values reported in the literature for a variety of commercially available soy protein products. Rat feeding studies employing hemoglobin repletion methodology have shown the mean relative iron availability from full fat soy flour (SF) to be 81% (Picciano et al., 1984); from soy protein isolate (SI) to be 61% (Steinke and Hopkins, 1978) and 82-100% (Rotruck and Luhrsen, 1979); and from soy protein concentrate (SC) to be 62% (Shah et al., 1983) and 92% (Picciano et al., 1984). Rat feeding studies employing radiolabelled test meals (Schricker et al., 1983), reported the relative iron availability from SF, SC and SI to be 70 to 90% that of ferrous sulfate added to casein-based diets. It would appear from the above mentioned literature values that
there is a general lack of ability on the part of the rat to discriminate between the availability of iron from different soy protein products, but that on the whole, the iron of soy protein is highly available.

20% of the iron in the soy product used in the present study was derived from ferrous sulfate, an inorganic iron source which is recognized as being highly available to the rat (Pla and Fritz, 1970). The effect of soy protein on the availability of this added iron was not investigated in the present study. However, as the RBV of the iron in the soy diet was high, it would appear that the effect of this particular soy product on the availability of the supplemental iron was minimal.

At the commencement of this study, the inhibitory effect of phytic acid on iron absorption was a highly controversial issue. Phytic acid, the hexaphosphate of myoinositol, is a strong chelating agent capable of binding di- and trivalent metal ions. This is particularly true of calcium, copper, zinc, magnesium and iron in vitro at physiological pH. However, mineral interactions in vivo are not clear and are complicated by protein-mineral phytate interactions (Erdman, 1979).

The origin of the postulate that phytic acid inhibits nonheme iron absorption is unclear, but it would appear to be largely based on the inhibitory effect of non physiological doses of purified sodium phytate on iron absorption in rats and in man (Rotruck and Luhrs, 1979). It is currently felt that the inhibitory effect of purified phytic acid is dependent on the chemical form and concentration (Morris, 1983). Recently, it has been reported that the utilization of iron by iron deficient rats was unaffected when sodium phytate was added to the diet at levels up to 4%
(Hunter, 1981). The present understanding in regard to the action of phytic acid on food iron availability is that the purified form and that of endogenous food phytate may differ considerably from each other (Rotruck and Luhrs, 1979; Morris, 1983).

In view of the current research and the excellent availability of iron in the soy product used in the present study, it is unlikely that the phytic acid content of the diet, present at the level of 0.33% (db), had an inhibitory effect on dietary iron absorption. Shah et al. (1983) found there was no difference in the bioavailability to rats of iron in either soy or rapeseed protein concentrate despite a three-fold difference in phytic acid concentration.

Human studies employing radiolabelled iron-containing meals have indicated that the iron of soybeans and various processed soy products is not highly available to this species. Reported mean ratios for soy iron: reference iron absorption include 0.17 for boiled soybeans and 0.33 for baked soybeans fed to infants (Ashworth et al., 1973); 0.15 for baked soybeans fed to adults (Cook et al., 1972); 0.10 for boiled soybeans fed to adults (Lynch et al., 1984); 0.18 for full fat soy flour, 0.35 for textured soy flour and 0.08 for soy protein isolate, all fed to adults (Cook et al., 1981).

The results of the present study as well as other rat data appearing in the literature indicate that the availability of iron of soy to rats is several-fold higher than that obtained in studies with humans. It would therefore appear that the anemic rat is not a suitable model for man in predicting the absolute availability of iron in such foods.
3. Beef-soy protein mixture

The RBV of iron in the meat-soy mixture (65%) was significantly better \( (P < 0.05) \) than that of the meat iron (56%), but it was significantly lower \( (P < 0.05) \) than that of the iron in the soy product (81%). Shah et al. (1983) repleted rats with a 2:1 (w/w) ratio of freeze-dried ground beef to soy protein concentrate product. These researchers also observed that the RBV of the iron in the beef-soy combination (62%) was significantly greater than the RBV of iron in beef (53%).

The absorption of nonheme iron in normal man was depressed by 61 and 53%, respectively, when a soy-extended beef patty, containing either a 3:1 or 2:1 (w/w) ratio of raw ground beef to unhydrated textured soy flour, was included in the meal (Cook et al., 1981). This observed decrease in nonheme iron absorption was attributed to a combination of the inhibitory action of soy and the reduction in the enhancing action of the meat. In the present study, despite the reduction in the proportion of beef protein in the beef-soy mixture over that in the diet containing only ground beef, the RBV of the iron in the beef-soy mixture was 17% higher than that of the iron in beef. Thus the promotion of iron absorption by the "meat factor", which is operative in man (Cook and Monsen, 1976; Hallberg et al., 1979), was not evident in the present study with rats. Similarly, the inhibitory effect by soy protein on nonheme iron absorption demonstrated in man (Cook et al., 1981; Hallberg and Rossander, 1982) was not observed in the present study. Furthermore, in man it has been shown that the addition of 100g ground beef to a soy isolate meal increased absorption nearly four-fold,
from 0.36 to 1.44% (Morck et al., 1982). In contrast, in the present study the RBV of the iron in soy was 25% higher than that of the beef-soy mixture. Based on the two examples given for man and the results of the present study, it appears that the effects of soy and beef protein, when present together on iron utilization in the anemic rat, are not the same as those observed in normal man.

F. General discussion

No definite statement can be made with regards to the effect cellulose powder might have had on the absorption of iron from the test source diets. Admittedly, the level at which this fiber source was added, ranging from 14 to 20%, mostly certainly exceeded the 5% recommended for most rat formulations (American Institute of Nutrition, 1977). A recent review by Reinhold (1982) of the effect of dietary fiber on iron bioavailability in anemic rats suggests that, at the level provided in the present study, some inhibition of hemoglobin regeneration would be expected. If the fiber did inhibit the absorption of iron, it presumably did so through one of two mechanisms. The fiber may have physically entrapped iron in the gut, thereby preventing potentially available iron from coming in contact with iron receptor sites in the intestine. Alternatively, iron that might have been absorbed in the large intestine could have been metabolized by the microbial flora of the cecum before reaching the colon (McCall et al., 1962; Reinhold, 1982).

A final comment is in order with regard to the methodology employed in the present study and the manner in which the RBVs of the iron in the test sources were calculated. The assay design involved repleting anemic
rats with a single level of iron provided by the test source and determining the potency of the iron source relative to that of the ferrous sulfate reference through the use of a graphic technique. However, the AOAC method (1980) stipulates that three levels of iron provided by both the test source and reference salt be assayed and that the responses for each be analyzed by a parallel lines technique. Amine et al. (1972) has commented that when RBVs are determined from a single level of test source iron, one is unable to determine the validity of the assay or the precision of the estimated potency. However, owing to the differences in the nutrient content of the test sources used in this study and the formulary objectives imposed, it would have been difficult indeed to design the diets to provide three levels of iron.

There is little information in the literature about the forms in which the intrinsic iron of soy occurs. Ellis and Morris (1981) have reported that a portion of the iron in soy may be present as monoferric phytate. In light of the differences observed in the availability of soy iron in rats and man, it would be useful if the chemical forms of iron could be determined for the various soy protein products available. Perhaps such information would assist in explaining the different iron availabilities observed between man and rat for soy protein as an iron source.
CONCLUSIONS

The objective of this research project was to assess whether the iron availability in a fortified textured defatted soy protein product or a mixture of beef and the fortified product was comparable to that of beef. The iron availability in these food sources was estimated by measuring the hemoglobin or hematocrit response of anemic rats following two weeks of repletion on diets supplying an equal level of iron from these sources. A relative biological value was assigned on the basis of the amount of iron furnished by reference ferrous sulfate that produced equal curvative responses.

The RBVs of the iron in the three diets, calculated on the basis of final hemoglobin and hematocrit values, were respectively: freeze-dried ground beef, 56% and 62%; fortified soy protein product, 81% and 79%; 2.3:1 (w/w) combination of beef and soy product, 65% and 68%.

The relative availability of iron in the three diets compared favorably to the values reported from rat studies employing similar methodology. Human studies, investigating the relative availability of iron in meat and soy protein products have demonstrated an opposite trend. In humans, the absorption of the iron in beef is comparable to or better than that of the reference source, while that of the iron in soy is at best one third of the reference standard. Human studies, using extrinsic radioiron techniques, have clearly demonstrated the inhibitory effect of soy protein on the absorption of other nonheme iron present in the same meal as well as the enhancing effect of beef protein on iron absorption.
Under the conditions of the present study with rats, neither of these dietary factors appeared to be operative. In the present study, judging from the high RBV obtained for the iron in the fortified soy protein product, it would appear that the effect of the soy protein on the availability of the iron in the soy product was minimal. The cellulose powder, present in high levels in all three diets, was more likely the significant dietary component inhibiting iron absorption; its mode of action presumably being one of physical entrapment.

While the original intent of this study was fulfilled, the limitations of the information obtained must be recognized. The difference between the two species with regard to their ability to utilize heme iron in beef and the iron contained in soy protein might limit the transferability of the information obtained in the present study to the human situation. These differences between the two species need further exploration, possibly through collaborative effort. The hemoglobin repletion technique does show promise, however, as a useful tool in determining factors that effect the absorptive process.
REFERENCES CITED


Layrisse, M., Martinez-Torres, C. and Roche, M. 1968. Effect of interactions of various foods on iron absorption.


Appendix A. Comparison of experimental and reported values for proximate analysis components.

<table>
<thead>
<tr>
<th>Analysis</th>
<th>Control sample</th>
<th>Experimental Value^1</th>
<th>Reported or Expected value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nitrogen (%, db)</td>
<td>Skim milk powder</td>
<td>36.69 (0.17)</td>
<td>37.29^2</td>
</tr>
<tr>
<td>Ether extract (%, db)</td>
<td>84 g mixture of skim milk powder, cellulose, glucose + 16 g corn oil</td>
<td>16.34 (0.62)</td>
<td>16.43</td>
</tr>
<tr>
<td>Neutral detergent fiber (% db)</td>
<td>cellulose powder (Alphacel)</td>
<td>97.92 (0.20)</td>
<td>99.00^3</td>
</tr>
<tr>
<td>Total iron (ppm, db)</td>
<td>raw ground beef</td>
<td>62.15 (0.30)</td>
<td>67.84^4</td>
</tr>
<tr>
<td>Magnetically extractable iron (ppm, db)</td>
<td>Fe^0, hydrogen reduced, 10 mg</td>
<td>1,000,000 (28284)</td>
<td>1,000,000</td>
</tr>
<tr>
<td>Phytic acid (% db)</td>
<td>Rapeseed meal</td>
<td>4.64 (0.10)</td>
<td>4.79^5</td>
</tr>
</tbody>
</table>

^1Mean ± (standard derivation) of 2 or 3 determinations.

^2Watt and Merrill (1963); item 369.

^3Polizzotto et al. (1983).

^4Watt and Merrill (1963); item 1328.

^5Experimental value reported by laboratory of Department of Foods and Nutrition, University of Manitoba, Winnipeg, Manitoba, through which sample was obtained.
### Appendix B. Growth and hematological responses for iron depleted rats fed test sources of iron during the repletion period.

<table>
<thead>
<tr>
<th>Diet treatment</th>
<th>Basal + 25 ppm iron (as FeSO₄·7H₂O)/Kg</th>
<th>Beef</th>
<th>Beef + Soy flour (2.3:1, w/w)</th>
<th>Soy flour</th>
</tr>
</thead>
<tbody>
<tr>
<td>BW initial (g)</td>
<td>85.2 ± 22.6a¹,²</td>
<td>127.0± 5.5c</td>
<td>116.2 ± 4.2c</td>
<td>100.8 ± 6.6b</td>
</tr>
<tr>
<td>BW final (g)</td>
<td>162.8 ± 29.7a</td>
<td>198.3± 12.6c</td>
<td>191.0 ± 17.9bc</td>
<td>177.7 ±12.9ab</td>
</tr>
<tr>
<td>Hb initial (g/dL)</td>
<td>3.5 ± 0.8a</td>
<td>3.1 ± 0.5a</td>
<td>3.1 ± 0.6a</td>
<td>3.1 ± 0.9a</td>
</tr>
<tr>
<td>Hb gain (g/dL)</td>
<td>6.6 ± 1.7b</td>
<td>4.6 ± 0.9a</td>
<td>5.7 ± 0.6b</td>
<td>7.7 ± 1.0c</td>
</tr>
<tr>
<td>Hematocrit initial (%)</td>
<td>13.2 ± 2.7a</td>
<td>15.3 ± 1.2b</td>
<td>15.4 ± 1.7b</td>
<td>15.5 ± 2.4b</td>
</tr>
<tr>
<td>Hematocrit gain (%)</td>
<td>22.4 ± 4.5b</td>
<td>15.3 ± 1.9a</td>
<td>17.3 ± 2.6a</td>
<td>22.3 ± 3.5b</td>
</tr>
</tbody>
</table>

¹Mean of 10 animals (± standard deviation).
²Means in a row followed by the same letter are not significantly different (P > 0.05) as determined by Duncan's multiple range test.
Appendix C. Growth, feed intake and hematological responses for iron depleted rats fed graded levels of iron as ferrous sulfate during the repletion period.

<table>
<thead>
<tr>
<th>Diet treatment</th>
<th>Basal</th>
<th>Basal + 5 ppm iron</th>
<th>Basal + 10 ppm iron</th>
<th>Basal + 15 ppm iron</th>
<th>Basal + 20 ppm iron</th>
<th>Basal + 25 ppm iron</th>
</tr>
</thead>
<tbody>
<tr>
<td>BW initial (g)</td>
<td>111.1 ± 20.0b(^1,2)</td>
<td>101.2 ± 20.2b</td>
<td>104.5 ± 12.8b</td>
<td>98.5 ± 13.6b</td>
<td>101.3 ± 21.4b</td>
<td>85.2 ± 22.6a</td>
</tr>
<tr>
<td>BW gain (g)</td>
<td>23.7 ± 9.1a</td>
<td>55.3 ± 10.6b</td>
<td>73.2 ± 10.8c</td>
<td>70.2 ± 18.4c</td>
<td>80.2 ± 14.3c</td>
<td>77.6 ± 16.3c</td>
</tr>
<tr>
<td>Feed intake (g)(^3)</td>
<td>131.4 ± 18.8a</td>
<td>157.3 ± 25.3b</td>
<td>188.3 ± 23.5c</td>
<td>184.4 ± 17.9c</td>
<td>195.8 ± 22.0c</td>
<td>181.8 ± 30.5c</td>
</tr>
<tr>
<td>Hb initial (g/dL)</td>
<td>3.8 ± 0.06a</td>
<td>3.6 ± 0.06a</td>
<td>3.6 ± 0.7a</td>
<td>4.1 ± 0.9a</td>
<td>3.5 ± 0.5a</td>
<td>3.5 ± 0.8a</td>
</tr>
<tr>
<td>Hb gain (g/dL)</td>
<td>-1.4 ± 0.6a</td>
<td>0.6 ± 0.7b</td>
<td>2.3 ± 0.9c</td>
<td>4.1 ± 0.9d</td>
<td>5.5 ± 1.1e</td>
<td>6.6 ± 1.7f</td>
</tr>
<tr>
<td>Hematocrit initial (%)</td>
<td>14.4 ± 2.3a</td>
<td>14.7 ± 2.6a</td>
<td>14.3 ± 2.4a</td>
<td>15.4 ± 2.4a</td>
<td>14.0 ± 1.9a</td>
<td>13.2 ± 2.7a</td>
</tr>
<tr>
<td>Hematocrit gain (%)</td>
<td>-2.6 ± 1.6a</td>
<td>3.2 ± 3.6b</td>
<td>9.0 ± 3.7c</td>
<td>15.4 ± 2.6d</td>
<td>19.4 ± 3.1e</td>
<td>22.4 ± 4.5f</td>
</tr>
</tbody>
</table>

\(^1\)Mean of 10 animals (± standard deviation).

\(^2\)Means in a row followed by the same letter are not significantly different (P > 0.05) as determined by Duncan's multiple range test.

\(^3\)Expressed on dry weight basis.