CHAPTER II

REVIEW OF LITERATURE

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Iron deficiency anemia is a widespread nutritional disorder affecting several hundred million people through out the world. The most commonly affected are the people from the economically disadvantaged sections everywhere. Nonetheless, it is not uncommon among the more affluent groups either. Our understanding of the factors leading to a deficiency of iron has greatly improved over the years and it is now known that a low intake coupled with poor bioavailability of iron is often the frequent cause of iron deficiency.

The factors that determine bioavailability of iron have been better defined in recent years enabling us to make some progress towards devising strategies for improving iron absorption. The focus of the present study is on developing a quantitative model (a regression equation) for assessing the adequacy of the present day vegetarian diets with respect to iron availability which takes into consideration the interaction effect of various enhancers as well as inhibitors present in these meals. A further objective is to use this equation to predict the quantity of enhancers required to achieve a defined level of iron availability from vegetarian meals containing known amounts of inhibitors.

Therefore the review of literature is limited to studies that are chiefly related to iron bioavailability and factors affecting it. It starts with a brief description of biological essentiality of iron to humans and its regulation through mainly,

the process of absorption. Following this the dietary requirements of iron as influenced by two major determinants, namely iron status of the individual and bioavailability of iron through diet are reviewed. The concept of iron bioavailability and absorption are discussed and various methods for measuring the same are outlined in some detail. Since the net bioavailability of iron in the diet is influenced by several factors, these are discussed individually and their role and mechanism in modifying bioavailability of iron are identified. The final section deals with the recent advances in quantifying and predicting the bioavailability of iron from meals.

Iron : Biological Essentiality

Iron is one of the essential trace elements that plays a vital role in many metabolic processes. Among them, the three important ones are, a) catalytic reactions in which iron participates in close association with certain enzymes such as aconitase which plays an important role in respiration via the Krebs' cycle, b) in all the redox reactions of significance, iron shuttles back and forth between its oxidised and reduced forms, namely Fe^{3+} and Fe^{2+} respectively and contributes towards generation of metabolic energy through the oxidative respiration process, and c) binding, transport and release of oxygen to the cells via hemoglobin. The chemical properties of iron in heme allow for easy association and dissociation of oxygen to the hemoglobin molecule, depending upon the concentrations of oxygen and carbon dioxide in the tissues. The same function is carried

out by iron within the muscle cells, in association with a functionally similar compound called myoglobin (Saltman et al, 1984).

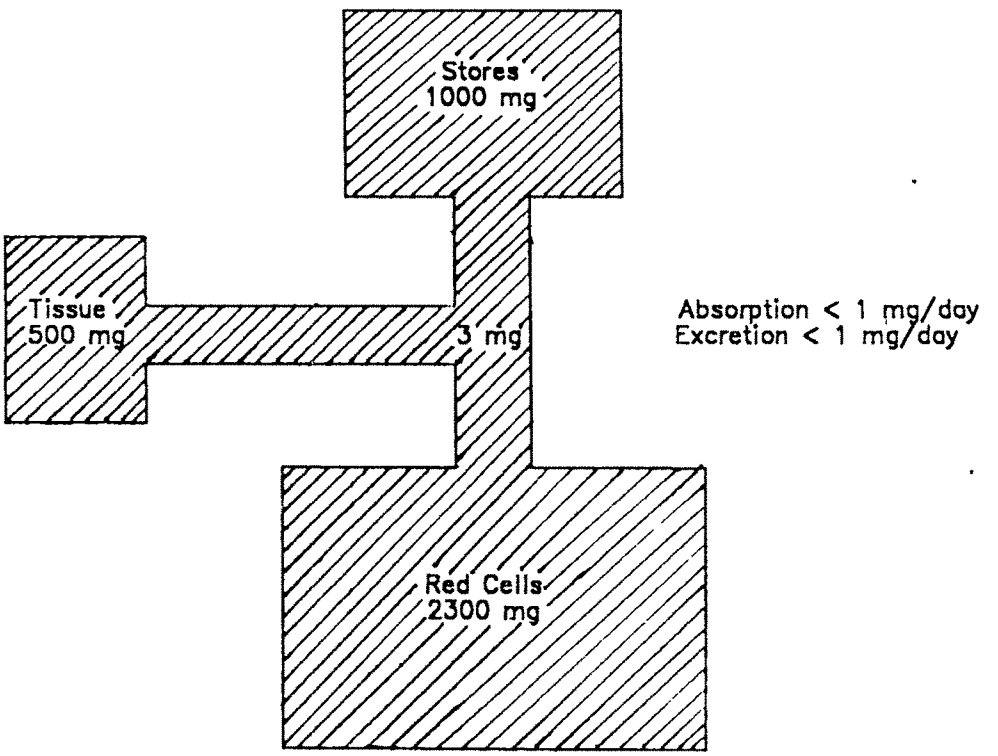
The iron status of a person is defined in relation to the amount of iron contained in two major compartments in the body - functional iron which includes the functionally active compounds namely, hemoglobin (Hb), myoglobin (Mb) and a variety of heme containing enzymes,and reserves, mainly present in the form of ferritin and hemosiderin in the liver, spleen, bone marrow and to a smaller extent in other tissues of the body.

At birth, a full term infant has a total of approximately 270 mg iron (Widdowson and Spray, 1951) while an adult has a total iron content of about 4-5g distributed among various compartments, as shown in Figure 1. Deposition of iron from infancy to adulthood, therefore, requires intake of adequate dietary iron in order to achieve the complete complement of this metal in the body.

Since humans have a markedly limited capacity to absorb dietry iron, there is a tendency for development of iron deficiency during prolonged periods of increased physiological demands such as early childhood, adolescence, pregnancy and lactation. In such instances, the storage iron gets depleted gradually to replenish the deficits in functional iron. As a result of depletion of iron stores, supply of iron to the tissues also becomes limited, resulting in some functional health consequences (Cook, 1990). Once absorbed, however, the human

Figure 1

The relative sizes of body iron compartments in a 75kg adult male. (Reproduced from Bothwell et al, 1979)



body conserves and regulates iron so efficiently that there is little or no excretion of the metal by the body.

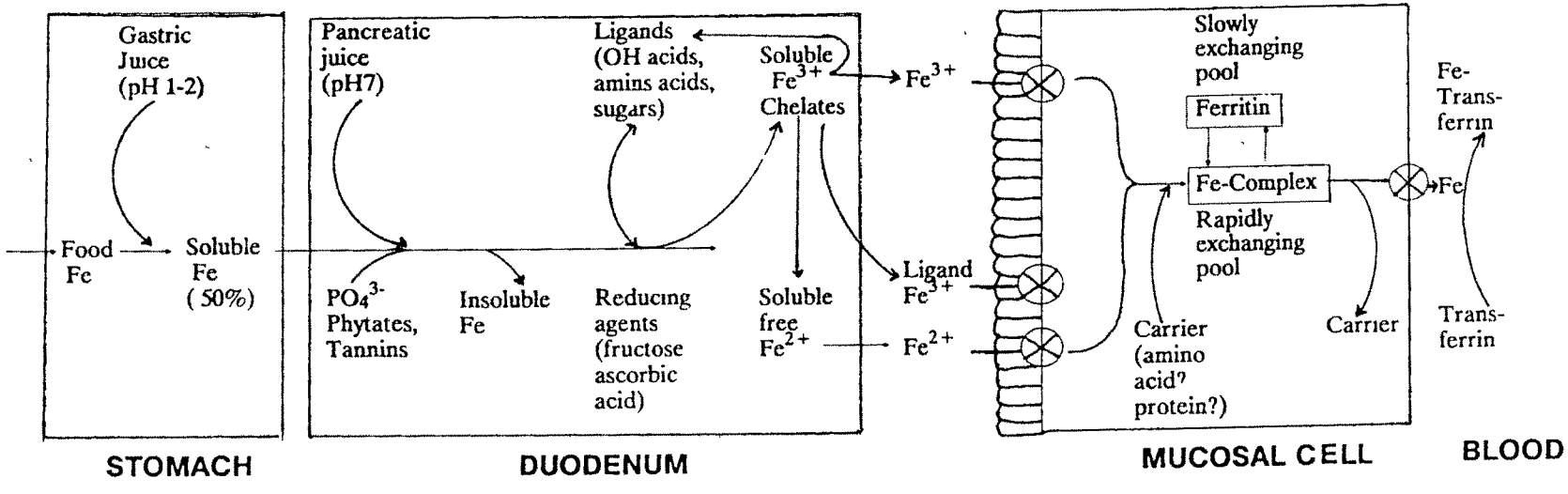
Iron Absorption and Regulation

Although studies in laboratory animals have suggested that absorption of iron can take place from any portion of the intestinal tract, the greatest proportion of food iron is absorbed from the duodenum and proximal jejunum (Moore and Dubach, 1962). The process of iron absorption is complex and can be divided into three main physiological phases (Figure 2). The first phase constitutes the intraluminal digestion of food by the action of gastric and pancreatic enzymes. Iron, which is usually present in a bound form in foods, is liberated first, by the action of gastric acid together with pepsin. As the liberated iron passes further in the lumen, it is attacked by a number of reducing agents and ligands, present concomitantly in the meal. Some of these ligands (enhancers) reduce the ferric iron to the soluble ferrous iron and chelate the iron, thereby retaining it in a soluble form even as the pH increases in the small intestine. Other ligands (inhibitors) may form insoluble complexes with iron thereby precipitating it at the alkaline pH of the intestine.

In the next phase, termed as the mucosal phase, iron is taken up by the mucosal cells and is further transported to the serosal side or retained as ferritin within the cells. In the final corporeal phase, the metal is bound to transferrin in

Figure 2

Schematic representation of the intestinal absorption of iron. (Reproduced from Narasinga Rao, 1981)



plasma and is carried to the liver and hemopoietic tissues for utilization and storage.

McCance and Widdowson (1937) showed that once iron is absorbed into the systemic circulation, little if any, of this metal is excreted through the urine, which led them to conclude that the amount of iron retained in the body is regulated by controlled rate of absorption. Since then, repeated attempts have been made to identify the exact mechanism of iron regulation in the body. Hann et al (1943) found that when dogs were fed a large dose of iron through diet, there was a block for subsequent absorption of a test dose consisting of radioiron,given one and a half hour later. Granick (1946) provided evidence for the "mucosal block" theory through his observation that in guinea pigs, there was an increase in the apoferritin content of the intestinal mucosa, immediately after iron feeding. This resulted in an increased formation of ferritin, which he believed was responsible for regulating the rate of iron absorption. Other studies using radio labeled iron postulated that a "ferritin curtain" in the columnar epithelium of the small intestine may act to regulate iron absorpition. Conrad and Crosby (1963) studied retention of ^{59}Fe in humans using a whole body counter. It was observed that although iron depleted individuals absorbed more iron (29% of the test dose) than the normals (<10% of the dose), they achieved iron equilibrium faster (3 to 7 days) than their normal counterparts (6 to 15 days). The authors postulated that a delay in iron loss in normal subjects could be due to retention of some of the absorbed iron in the epithelial cells.

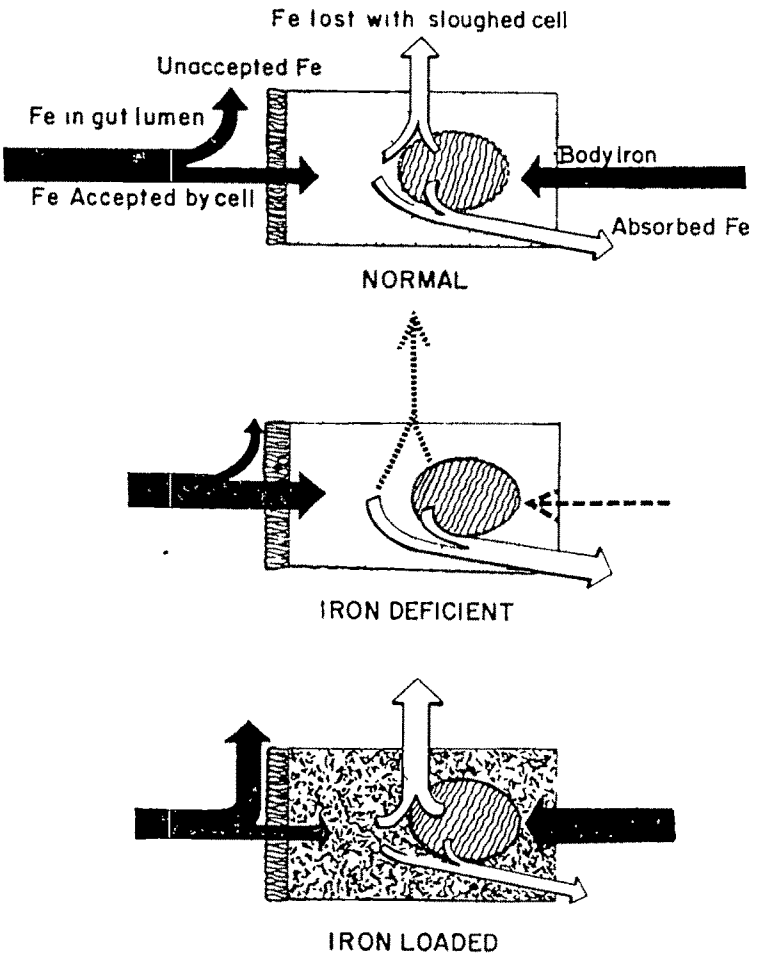
Only when those cells were sloughed off, could their iron be lost from the body, as illustrated in Figure 3. In iron deficient subjects, on the other hand, there was no retention of ^{59}Fe in the mucosal cells and hence they could achieve equilibrium faster (i.e. during the time when the unabsorbed iron was excreted through the intestines). The authors further studied the radiographs of duodenal and jejunal sections of rats, after injecting ^{59}Fe in these segments. It was found that there was no radioactivity in the columnar epithelial cells of iron deficient or iron loaded rats. They ascribed this to the fact that while in iron deficient animals most of the absorbed iron was immediately chanelled for utilization and not retained within the cells, in the case of iron loaded animals, most of the iron was excreted as the cells were already loaded with ferritin.

However, in the case of normal rats, some radioactivity was detected in the mucosal cells, indicating that some amount of the absorbed radioactive iron was retained in the epithelia of these rats. These obsrvations led the authors to hypothesise that the "ferritin apparatus" of the epithelial cells played an important role in regulating day to day control of iron balance (Crosby, 1963).

The mucosal block theory was held as valid for sometime. However, several other lines of evidence have led to its rejection. Brittin and Raval (1970) measured the incorporation of labeled leucine into ferritin of iron deficient and iron replete rats, before and after intraduodenal administration of

Figure 3

A concept of control of iron absorption by the intestinal mucosa, in normal, iron deficient and iron loaded subjects. (Reproduced from Conrad and Crosby, 1963)



radioactive iron. They demonstrated that though prior to iron dose, there was little ferritin in the duodenal homogenates of iron deficient rats, after iron administration there was rapid synthesis of ferritin and within five hours they contained more ferritin than that present in the duodenum of iron replete rats. However, when a second dose of iron was given to the iron deficient rats, they still absorbed significantly higher amount of iron (14%) than their normal counterparts (1.6%) eventhough the ferritin content of both the groups was similar. These results argued against the mucosal block theory,for if ferritin content of the cells could block further absorption of iron, there should not have been an appreciable absorption of the second dose of iron in iron deficient rats.

These studies led the researchers to explore other modes of regulatory mechanisms of iron absorption. One of the possibilities could be the presence of specific iron receptors at the brush border of the mucosal lining. Indeed, receptors that are iron specific have been identified in human biopsy sections of the duodenum and ileum, using flourescently labeled antibodies (Banerjee et al, 1986). This study revealed that there was an increased iron receptor population in iron deficient subjects as compared to the subjects with normal iron stores, thereby providing some evidence for their involvement in the regulatory mechanism. However, the biopsy sections of human colon, where little absorption takes place, also showed presence of these receptors while in the duodenal section of patients with hemochromatosis, where iron absorption is known to be increased,

these receptors were absent. This led to the postulation by the authors, that the brush border receptors may not be directly involved in the process of mucosal uptake or tansfer of iron.

Savin and Cook (1980) suggeted the hypothesis that the signal which determines how much iron needs to be absorbed by the body, probably lies within the intracellular milieu of the mucosal cells. The authors studied the relationship between iron absorption and concentration of transferrin and ferritin in the duodenal mucosal cells, using immunoradiometric assays. The findings demonstrated that there was an inverse correlation between mucosal ferritin and iron absorption, while mucosal transferrin varied directly with the uptake as well as transfer of radioiron by the cells. The ratio of transferrin to ferritin, however, exhibited the highest correlation with iron absorption. The authors suggested, therefore, that there may be two distinct compartments within the mucosal cells; storage compartment containing ferritin and transport compartment containing transferrin. They postulated that a balance between these two iron binding proteins in the intracellular environment probably controls the rate of iron absorption. In fact, an iron binding protein, similar to serum transferrin has been identified and purified from the proximal mucosal fractions of guinea pigs (Pollack and Lasky, 1976). It has been demonstrated by the authors that this new intracellular protein could bind, maximally, the ferric iron at approximately two moles of iron per mole of the protein. The authors also suggested that since the affinity of this protein for iron was much lower than that of

serum transferrin, transfer of iron from the mucosal cells to the plasma cells could be possible thermodynamically. This implies that the intracellular, transferrin like protein, may be involved in some way, in iron transport in the mucosal cells.

The exact nature of the mucosal transferrin is not very clear. However it is suggested that it differs from serum transferrin slightly, in the amino acid composition and iso-electric point (Huebers et al, 1983). The authors also commented that it may be synthesized by the mucosal cells themselves or secreted by the liver in bile. Further research is required, however to pinpoint the exact machanism which limits the rate of iron absorption in human body. Though studies on iron stores (Charlton et al, 1977), rate of erythropoiesis (Weintraub et al, 1965) and hypoxia (Hathorn, 1971) have all contributed towards a greater understanding of the mechanism of iron regulation, the exact signal is yet to be identified.

Dietary Iron Requirements

The requirement for dietary iron arises from three major needs of the body : endogenous loss of iron, requirement for growth and building up of iron stores over the period from infancy to adulthood.

It has been demonstrated by several investigators, using radioisotopic iron in animals and humans, that a certain amount of iron is lost daily from the skin, the gastrointestinal tract and the genitourinary tract, together termed as the obligatory

loss of iron (Hann et al, 1939; Dubach et al, 1955). It has been summed up, based on individual fractions of endogenous losses, that an adult man may lose between 0.5 to 1.0 mg iron daily while an adult premenopausal woman may excrete an additional 0.5 to 1.0 mg iron daily, due to loss of blood during menstruation (Moore and Dubach, 1962).

Although these losses seem to be of a very small magnitude, in view of the fact that only a fraction of dietary iron is actually made available to the body, it becomes imperative to cover these losses through adequate dietary intake of iron. In order to maintain normal iron balance, an adult man needs to absorb a minimum of 0.9 mg iron daily while in the case of woman, the need for absorbable iron ranges from 1.2 to 1.4 mg per day (Monsen, 1988).

Considering 2 to 5% absorption of dietary iron from cereal based vagetarian meals, as is the general meal pattern in India and other South East Asian countries, at least 20 to 30 mg dietary iron is required to meet the requirements of absorbable iron in an adult man and woman (ICMR, 1989a).

This is because the cereal meals that are habitually consumed in India are low in non-vegetarian animal foods such as meat, fish, and poultry as well as low in ascorbic acid rich foods, which are known to enhance iron absorption. Presence of these enhancers in appreciable quantities in typical Western meals results in 10 to 20% absorption of dietary iron, which

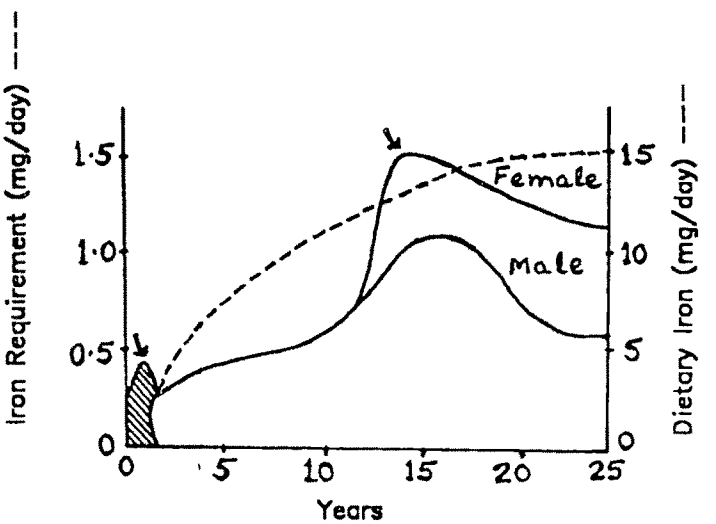
decreases the total dietary iron requirements for adults to 10 to 15 mg iron per day (Viglietti and Skinner, 1987).

The amount of iron that is needed to support growth is usually calculated from the difference between the body iron content at birth and in an adult. The requirement for iron at various ages and the critical periods where intake and losses are of similar magnitude are shown in Figure 4.

As can be seen from Figure 4, the requirements for iron are highest during infancy when the iron stores are completely depleted and the rate of growth is at its peak, resulting in a negative iron balance. This is because milk, the major dietary source of iron during the first year of life, is very low in iron content and cannot meet the increased demands of the body for this mineral. The second critical period, with respect to iron, occurs during adolescence, especially in females, when the loss of iron due to menstruation imposes greater needs for intake of dietary iron. During these two critical periods of life, the chances of developing iron deficiency anemia are high. Anemia is widely prevalent in the Indian population, especially in pregnant women and children below the age of six years, though its prevalence is not uncommon among other sections such as adult men (ICMR, 1989b). However, the daily intake of iron through the diet ranges from 30 to 32 mg by an average adult man or woman (non pregnant, non lactating) (NNMB, 1984), which is close to the recommended dietary allowance of iron for adults (ICMR, 1989a). These observations raise the question as to why iron deficiency anemia is so widely prevalent inspite of a near-

Figure 4

Iron requirements in man. The continuous line indicates the iron requirements. The shaded area indicates a period of negative iron balance. The arrows indicate two critical periods when iron deficiency is likely to develop. Dotted line indicates availability of iron from a normal 'Western' diet. (Reproduced from Bothwell et al, 1979).



adequate dietary iron intake. The concept of bioavailability, therefore, comes into picture. We know now, that it is not only the total iron content of the diet which is important but also the amount of iron made available to the body from the diet. Besides, availability of iron from a composite diet may not be the same as that from the sum total of individual foods taken separately (Hallberg, 1974). This is because there is a significant interaction between different constituents of food which in turn influences the bioavailability of iron.

The Concept of Bioavailability

There is some controversy regarding the exact definition of this term, in the context of assimilation of iron by the body. Since most of the available methods for measuring iron absorption do not differentiate between the fraction of this nutrient that is absorbed and the fraction which is actually utilised by the body, the term absorption has been taken, very often, to mean bioavailability. The arguement for using these two terms synonymously is that once iron is absorbed, it is eventually going to be utilised or retained within the body. Therefore, iron absorption has been used as a measure of iron bioavailability in the literature. Attempts have been made, however, to distinguish between these two physiological terms by defining them in a more explicit manner. It has been suggested by O'Dell (1985) that bioavailability must refer to that fraction of iron in food which is absorbed and utilised by the body. Utilization includes the process of transport, cellular

assimilation and conversion to a biologically active form. Absorption, on the other hand, refers to that portion of a nutrient in food, which moves from the intestinal lumen through the mucosal cells into the body.

Further confusion arises by the use of an additional term 'in vitro iron availability' which is used to express results of studies based on the in vitro techniques. Since the in vitro methods provide only a directional evidence rather than giving an absolute indication of iron bioavailability, it has been suggested by Hallberg (1985) that the term 'available iron' be used to describe the iron measured by this technique which is the amount of iron in a meal that is "potentially" available for absorption. The term 'bioavailable iron', on the other hand, should be used only when iron from a meal is actually absorbed by subjects of a known iron status. Hence, in this thesis, 'bioavailable iron' is used to refer to in vivo absorption or utilization of iron, as reported by various investigators while the term 'available iron' is used to describe results of studies which have used the in vitro techniques.

As mentioned before, absorpiton of iron from a meal is often taken as a measure of iron bioavailability. However, at times, it becomes necessary to express the amount of iron absorbed per unit of energy consumed by the subjects, to facilitate comparisons between meals. The concept of bioavailable iron density has been evolved, therefore, by Hallberg (1981). According to him, bioavailable iron density can be defined as the

amount of iron absorbed from a meal per unit energy consumed (usually taken as 1000 Kcal). This concept helps in judging whether manifestation of iron deficiency anemia in a particular segment of the population is related to an overall inadequate dietary intake or due to poor bioavailability of iron per se, in the diet. This is especially relevant to the Indian situation where a low total calorie intake may be responsible, partially, for a low iron status of certain vulnerable groups of the population, such as pregnant women (Prema, 1984). Hence relating the bioavailability of iron with calorie intake assists in deciding whether supplementary feeding alone can bring about an improvement in iron status of these women or bioavailability of dietary iron also needs to be improved.

In view of the fact that measurement of iron absorption or iron bioavailability is a more precise measure of dietary iron adequacy than the total iron content of the diet, several methods have been developed over the years, to determine these parameters in humans and animal systems. Some of the important methods are described in the next section, which is followed by a review of the in vitro methods for determining iron availability.

Methods for Estimating Iron Bioavailability

In general, two main approaches have been used for measuring iron bioavailablity over the years. In earlier studies, non isotopic chemical balance technique was used while in reccent years, it has been largely replaced by radio-isotopic techniques.

Chemical balance

Some of the earliest classical observations regarding iron absorption and excretion have been made by using the chemical balance technique (McCance and Widdowson, 1937). The method involved oral feeding of known amount of iron over a period of twenty four hours, and precisely measuring the excretion of iron through feces, over a period of two to three weeks. The difference between the quantity of iron ingested and the amount of iron excreted was expressed as a measure of iron absorption. Till date this method is the only one which can measure iron absorption from a varying diet, over a period of several days or even weeks. However, from a technical point of view, it becomes difficult to adhere to a high degree of accuracy in measurements because the amount of dietary iron absorbed is minute as compared to the total iron content of the diet and the iron excreted through feces. Also, the procedure is tedious and cumbersome. Despite these limitations, this method found wide application in early classical studies such as those carried out by Widdowson and McCance (1942) where iron exchanges of adults were investigated using white and brown breads. Based on their findings, using the above technique on eight human volunteers, the authors observed that more iron was absorbed form white bread than from a bran rich, brown bread. This classical finding inspired further research on the role of bran and phytate, in modifying iron absorption in humans. With the recent development of isotopic methods, however, chemical balance method has become chiefly one of historical interest.

Radioiron balance

This method owes its origin to Dubach et al (1955) who studied iron excretion in nine human subjects, using parenteral doses of radioactive iron. Excretion of tracer iron through feces was then estimated for 5-day periods, at different intervals, during a total of 140 days. Based on their estimations, the authors calculated the average loss of iron through feces in a normal adult, to be ranging from 0.3 to 0.5 mg daily.

Since then this method has been extensively used for both, iron excretion and iron absorption studies where a known amount of tracer iron is ingested orally and its excretion through feces is recorded over a period of two weeks or more. Use of fecal markers such as carmine red dye or brilliant blue dye ensures complete collection of the feces.

The use of radioiron balance method calls for a high degree of co-operation from the subjects and is expensive in terms of cost of materials and equipment required. Therefore, in recent years, this method has given way to some simpler and accurate methods such as hemoglobin repletion technique and red cell incorporation of the ingested iron.

Hb repletion method

In anemic subjects, practically all dietary iron is utilized preferentially for regeneration of Hb and therefore a rise in Hb

on iron supplementation reflects a measure of iron bioavailability in these subjects. Based on this principle, Hb regeneration technique was applied by Fritz and Pla (1972) to assess the availability of iron from foods using an animal model. This method found use conveniently with rats and chicks since they could be made anemic faster by a combination of phlebotomy and low iron intake through diet. Once they were anemic, the test food was fed to these animals and the responce in Hb regeneration was evaluated at the end of two weeks. A relative biological value was assigned to each food or iron compound tested, in comparison to the reference compound ferrous sulfate. In a recent task force report of the INACG (Forbes et al, 1989) the Hb repletion technique was recommended as a promising indicator of bioavailability.

Red cell radioiorn

A unique feature of iron absorption is that most of the absorbed iron is normally incorporated into circulating red cells within 7 to 10 days of oral administration. Several investigators have used this principle to measure iron absorption in man (Cook et al, 1981; Hallberg and Rossander, 1982). The procedure involves ingestion of the radioiron, either extrinsically tagged or intrinsically incorporated into the test food as described later. Background radioactivity is measured in the blood one to five hours after ingestion of the test food. After two weeks, blood is again drawn to measure the incorporated red cell radioactivity. Absorption of iron is then calculated on

the basis of blood volume (taken as 65 ml/kg body weight) and the erythrocyte incorporation, assumed to be 80% of absorbed iron (Lynch et al, 1984; Forbes et al, 1989). The assumption of 80% incorporation, however, may not hold valid in the case of patients with abnormalities in the absorptive mechanism, who have lower % incorporation of radioiron into red cels e.g. aplastic anemia, hemolytic anemia, iron overload and ineffective erythropoiesis (Bothwell et al, 1979).

For incorporating the radioiron into the food, two basic approaches have been used, intrinsic tagging and extrinsic tagging. In the early studies, such as those by Moore and Dubach (1951), radioactive iron was incorporated into the foods by either growing the vegetables in nutrient solutions containing the isotope, or in the case of eggs and other animal products (liver, muscle) by injecting the radioiron into hens or rabbits. These intrinsically labeled foods were then fed to fasting subjects.

Later it was demonstrated by Cook et al (1972) and Layrisse and Martinez-Torres (1972) that use of tracer amounts of extrinsic tag (radioiron, mechanically and homogeneously mixed with the test food) behaved in a fashion, identical with the nonheme iron present in the meal. These observations led to the extensive use of extrinsic tagging of entire diets, for measuring iron absorption. A significant advance in the validation of the extrinsic tag technique came from the observation of Hallberg (1981) who demonstrated that both intrinsic and extrinsic tagging of food gave the same measure of iron absorption in human

subjects. This indicated that extrinsically added radioiron was able to mix uniformly with the entire pool of iron and hence could be used, with a fair degree of accuracy for measurement of iron bioavailability from composite meals.

An alternative to the measurement of incorporation of radioiron in erythrocytes is whole body counting. In recent years, this approach has been used extensively in the in vivo human situation (Hallberg et al, 1987). The method involves administration of the test meal containing radioiron to the subjects after an overnight fast and measurement of background activity, one to five hours later. After 14 days, a second measurement is obtained to determine the relative absorption of the tracer, by whole body counting.

Use of reference dose

One of the major limitations with iron absorption studies using red cell incorporation or whole body counting is that values vary widely for different individuals depending upon their iron status, and therefore a comparison of values obtained for the same meal becomes difficult in subjects with varying iron stores. In order to eliminate this variation Layrisse et al (1969) proposed the use of a reference dose of iron and expressing the absorption of iron from a meal for each individual as a ratio of the reference dose. Such a measurement is largely independent of the iron status of an individual. By general consensus the reference dose has been defined as consisting of 3

mg elemental iron in the form of ferrous ascorbate, containing 2:1 molar ratio of ascorbic acid to iron. It has been demonstrted by Magnusson et al (1981) that there is 40% absorption of this reference dose in borderline iron deficient subjects i.e. subjects with no iron stores and who have yet not developed anemia. It is a general practice, therefore, to adjust all food iron absorption values to 40% reference dose value. The procedure involves administration of the reference dose, given as a drink (labeled with ^{59}Fe) and measurement of readioactivity after 14 days, in order to obtain a measure of each subject's capacity to absorb iron. The absorption of iron from a given test meal is then calibrated as follows (Gillooly et al, 1983).

$$\text{Corrected food iron absorption} = \frac{\text{Calculated food iron absorption in a subject}}{\text{Calculated reference iron salt absorption in the same subject}} \times 0.40$$

Use of iron absorption values, which have been calibrated in the above manner, provide a more sensitive indicator of the relative bioavailability of iron from different meals than expressing the absolute values per se.

Use of double isotopes

With the advent of reference dose absorption measurement for individual subjects, studies using only one radioisotope required twice the period of time for completion of both test food and

reference dose absorption measurements. This led to the development of double isotope technique. In this method, two different tracers, namely ^{59}Fe and ^{55}Fe could be used simultaneously to label the test food and the standard reference dose respectively. Hence measurement of radioactivity in the blood, of both the tracers could be carried out at the same time. Besides, by use of sequential ^{55}Fe and ^{59}Fe labels, measurement of iron absorption could be done for four separate test meals in an individual subject. Therefore in most of the recent studies double isotope technique is being used for measuring iron absorption from various test meals (Cook et al, 1991).

Use of stable isotopes

In order to avoid exposure of racdioactive material to the subjects, use of stable isotopes has been encouraged recently (O'Dell, 1985). These can be used safely, in all segments of the population and have the advantage of no decay with time. However very high cost and limited availability of the stable isotopes poses some constraint over their use. Also, the analytical procedure requires meticulous skill and training on the part of the investigator. Besides, the amount of isotope used extrinsically to label the food is too large to be considered a tracer and hence it adds to the total quantity of the mineral in the food, appreciably. In view of these limitations human studies which employ the use of stable isotopes are fewer as compared to those which make use of radioactive tracers.

From the discussion of the in vivo techniques for measurement of iron absorption, it is evident that meal samples spiked with small amount of radioiron may be used effectively for an accurate assessment of dietary iron bioavailability. However, in view of the expensive and time consuming nature of these in vivo methods, techniques have been developed which meausre iron availability in the 'in vitro' system, by simulating some of the events of gastrointestinal digestion, liberating the absorbable iron and its colorimetric estimation. These offer an appealing alternative to the 'in vivo' method, especially for screening a wide variety of foods for their iron availability.

In vitro techniques

In vitro iron availability studies depend on the in vitro digestion of foods and additives under conditions simulating human digestion. This generally includes digestion at gastric pH (pH 1to2) in the presence of pepsin and subsequently at the intestinal pH (pH 6to7) in the presence of pancreatic enzymes. The state of iron is then ascertained by dialysis to measure low molecular weight iron (Miller et al, 1981) or by a combination of centrifugation and the use of ferrous iron chromogen to test for iron in the supernatant (Narasinga Rao and Prabhavati, 1978; Kojima et al, 1981).

Advantages of in vitro methods include simplicity, low cost and higher efficiency of the procedure to screen a wide variety of foods or diets, within a limited period of time. Also, these

techniques give the opportunity to directly monitor the interaction of iron with other nutrients under digestive conditions.. The in vitro methods can also be used to determine the extent to which availability of iron can be improved by manipulation of diets.

However, such methods which use the physiological approach for simulating the actual digestive process, do not take into account the humoral and mucosal factors which significantly influence iron absorption in individuals. Therefore the values obtained by the in vitro methods must be viewed as relative rather than absolute indications of availability.

A key to the acceptance of the in vitro availability studies is the validation of the in vitro techniques used. Although some of the early methods proposed were not altogether satisfactory (Jacobs and Greenman, 1969; Ranhotra et al, 1971), more recently Narasinga Rao and Prabhavati (1978) as well as Schricker et al (1981) have demonstrated substantial agreement between the in vitro iron availability and in vivo iron absorption values in human subjects. Schricker et al (1981) also found that the in vitro method correlated better with the in vivo measurements than the rat assays. Hence they concluded that because of physiological difference the availability of food iron to humans was more accurately estimated with in vitro measurements than with rat studies.

Recently a task force report of INACG (Forbes et al, 1989) on comparison of in vitro, animal and in vivo human

determinations of iron bioavailability recommended the in vitro dialysis as a promising screening technique for predicitng iron availability for humans. The group further suggested that although animal studies may serve as useful predictors in practical terms, it should be recognised that the human diet is mixed, containing components that interact together to influence iron absorption. The experimental diets in animal studies, on the other hand, are comprised of ingredients that are in purified form, hence are more homogenous in nature. In view of this, in vitro studies which can be performed at a small fraction of the cost of animal studies can probably produce results comparable to those obtained in the in vivo studies. However, in vitro methods need precise standardization since even relatively small variations in incubation time can lead to pronounced differences in estimated in vitro available iron (Forbes et al, 1989).

Inclusion of pancreatic secretions in the in vitro methods is thought to give a better estimate of iron availability since it is speculated to be responsible for the formation of unavailable iron hydroxide polymers due to the presence of bicarbonates in bile. However, it is possible that this effect of bicarbonates may be counter-balanced by the presence of cleaving enzymes that may release substances such as amino acids and small peptides from food which serve as ligands for enhancing iron absorption (Bothwell et al, 1979). Therefore the net effect, on the in vitro availability of iron, by including pancreatin in the incubation mixture may not be significant, for final interpretation of the data. This was experimentally demonstrated

by Narasinga Rao and Prabhavati (1978) who explored the effect of including pancreatin during the incubation of food at pH 7.5. The findings revelead that pancreatin addition had no effect on the final values obtained for soluble and ionisable iron.

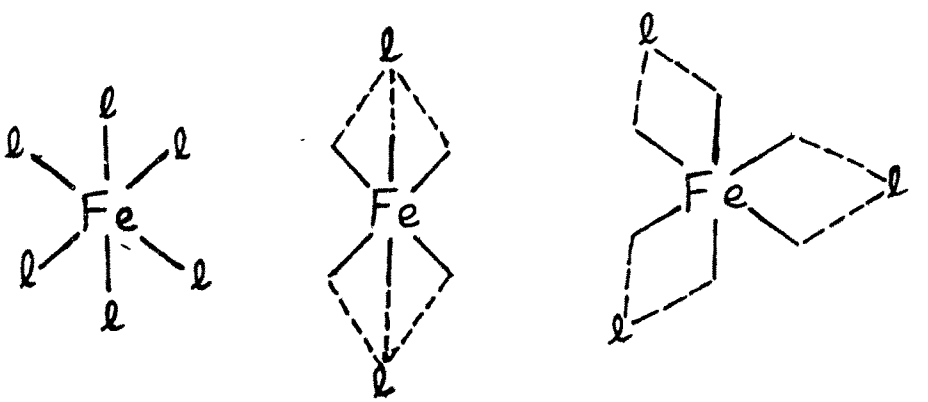
Formation of Iron Complexes and Their Solubility

Some knowledge of the chemical properties of iron is essential to understand the behaviour of iron in foods, during digestion and the influence of various factors on its bio-availability. Iron can exist in several states of oxidation from Fe^{6+} to Fe^{2-}. However, the most common states of iron in foods are Fe^{3t} and Fe^{2+}. Both forms of iron are soluble at the acidic pH of the stomach. However as the pH is varied, the Fe^{3+} becomes much less soluble than the Fe^{2+}. The solubility of Fe^{3+} at pH 7 is 10^{-18} M whereas that of Fe^{2+} at the same pH is 10^{-1}. Thus the bioavailability of iron can be much higher if iron remains in Fe^{2+} state at the somewhat alkaline pH of the small intestine where most absorption takes place. In this context, reducing agents like ascorbic acid have an important role in modulating iron availability.

In acidic acquous solutions the ionized iron (i.e. Fe^{3+} and Fe^{2+}) does not remain in the free form but is usually hydrated as $[Fe^{3+}(H_2O)_6]$ and $(Fe^{2+}(H_2O)_6)$. As the pH rises, the protons are split off (i.e a process of hydrolysis) resulting in the formation of hydroxides, $Fe(OH)_3$ and $Fe(OH)_2$ that become increasingly insoluble if pH is raised and also the process of hydrolysis becomes irreversible, thus tending to make the iron

unavailable. However, formation of different types of complexes as the pH is raised greatly alter the availability of iron, some making it more soluble than the others, thus providing the means to enhance iron absorption.

In general, metal ions can form bonds with other compounds called ligands. The number of possible bonds with a ligand is determined by the co-ordination number of the metal, which for iron is 6. The complexes thus formed can be monodentate (six lignads forming one bond each with one iron atom), bidentate (two lignads each forming three bonds with one iron atom) or multidentate (three lignads each forming two bonds with one iron atom) as shown below. The multi dentate complexes are more stable than the monodentat complexes.



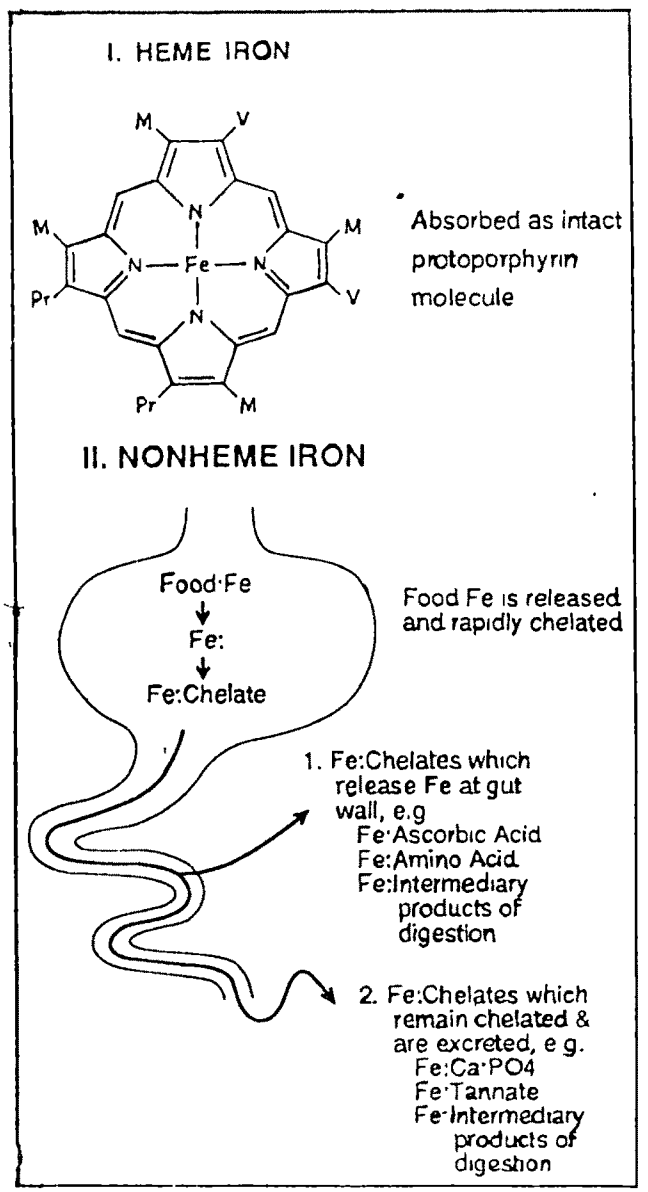
The relevance of this to iron bioavailability resides in the thermodynamic and kinetic stability constants of the complexes and the interfering reactions with other legands. As the iron or iron complexes enter the intestinal milieu they can dissociate and reform complexes, the nature of which will depend upon the concentration and affinity of the competing ligands for the metal. The ligands which have a high affinity for iron and form insoluble complexes make iron unavailable (inhibitors) while the lignads that form stable soluble complexes make the iron more available (enhancers). More details about individual complexing agents are described in the next section.

Dietary Factors Affecting Iron Availability

There are two major chemical forms of iron in a mixed diet, and each is absorbed by a different mechanism (Figure 5). Heme, containing iron in a porphyrin ring structure, is found in hemoglobin and myoglobin and accounts for nearly 40% of the iron present in animal tissue including fish and poultry (Monsen et al, 1978). Because of the porphyrin complex the heme iron is protected from the action of complexing or reducing agents present in the meal and is taken up by the mucosal cell as the intact protoporphyrin complex. Absorption of heme iron is therefore high, averaging from 15% in iron replete individuals to 35% in those lacking iron stores (Monsen, 1988). Once absorbed by the cells heme iron is split from the porphyrin ring by the

Figure 5

Schematic diagram of the absorption of the two forms of food iron; heme iron and nonheme iron. (Reproduced from Monsen, 1988)



action of a specific enzyme - xanthine oxidase (Dawson et al, 1970). This enzyme also probably plays a role in promoting the oxidation and incorporation of iron into mucosal transferrin (Topham et al, 1982). Non-heme iron and heme iron then seem to have a common pathway from the mucosal cell to the plasma.

The other chemical form of iron, namely, nonheme iron is largely present in foods of vegetable origin and in noncellular animal foods such as eggs and dairy products. In addition, approximately half of the iron in meat, fish and poultry is nonheme. The specific rate of nonheme iron absorption depends upon the iron stores of the individual as well as on the dietary constituents ingested concomitantly (Morck and Cook, 1981). Hence, absorption of nonheme iron ranges widely from 2 to 20% depending upon the above two determinants (Monsen, 1988).

In the past two decades, a number of factors have been identified, that influence nonheme iron absorption in varying potencies (Table 1) which are discussed individually in the following section.

TABLE 1

Food components affecting dietary iron bioavailability

(Reproduced from Morck and Cook, 1981)

Food components	Potency
Enhancers	
Ascorbic acid	+++
Animal tissue	+++
Inhibitors	
Tea	+++
Soy products*	+++
Coffee	++
EDTA	++
Calcium/Phosphate salts	++
Eggs	
Yolk	++
Albumen	++
Wheat bran	++
Phytate	±
Fibre	±

* When the major proportion of dietary protein is provided by soy.

Enhancers

Absorbic acid

The initial clue to the enhancing role of ascorbic acid came from the studies of Moore and Dubach (1951) which was later confirmed by others who used crystalline form of ascorbic acid (Sayers et al, 1974; Cook and Monsen, 1977).

The studies available in the literature on the effect of ascorbic acid on iron absorption/availabiltiy are summarised in Table 2. These include studies that have used crystalline ascorbic acid as well as foods rich in ascorbic acid, and in vivo as well as in vitro studies.

Major findings in vivo : As can be seen from the Table, the diets tested in the in vivo situation comprised of both low iron availability diets (availability ranging from 1.2 to 4.0%) and high iron availability diets (iron availability more than 9.0%). The iron content of the meals in all the studies ranged from 3 to 6.2 mg. The levels of ascorbic acid tested varied from as low as 15 mg to as high as 1000 mg does of the vitamin. The molar ratios of ascorbic acid to iron ranged from less than 2 to 75.6.

One of the major findings that emerges from the Table is that ascorbic acid enhances iron absorption from both low and high availability meals, added in crystalline form or native food form to thee meals. The magnitude of increase, however is much higher in the low availability nonheme iron containing meals than in the high availability heme containing meals. The increase in absorption that occurs with ascorbic acid is clearly related to

TABLE 2

Effect of ascorbic acid on iron absorption/availability in the in vivo and the in vitro systems

	Reference	Type of study	Basal meal and iron content	Form quantity of ascorbic acid (mg) and molar ratio of ascorbic acid to iron	Effect on iron absorption/ availability	Direction and quantum of increase
1.	Apte and Venkatachalam, 1965	In vivo	Cereal based vegetarian meals	Crystalline 200 mg (in two doses)	Fecal iron loss decreased from 77-93% to 29-81% of the total iron intake	-
2.	Callender et al, 1970	In vivo	Bread, egg and tea or coffee	Orange juice	3.7 to 10.4%	Three fold ↑
3.	Sayers et al, 1974	In vivo	Rice and dal (6.2 mg)	Crystalline 35 mg (1.76)	4.4 to 6.6%	Non significant increase
			Rice and vegetable soup (5.1 mg)	Crystalline 60 mg (3.67)	3.2 to 11.9%	Three and a half fold ↑
			Rice and vegetable soup (5.1 mg)	Crystalline 60 mg (6.07)	4.2 to 12.2%	same as above
4.	Layrisse et al, 1974	In vitro	Maize meal (3 to 4 mg)	Crystalline 70 mg (5.4)	1.38 to 7.9%	Five fold ↑
				Papaya (66mg) (5.1)	1.38 to 8.7%	Seven fold ↑
5.	Derman et al, 1977	In vivo	Maize porridge (2 mg)	Crystalline 0 50 mg (7.7) 100 mg (15.5)	3.8% 34% 35%	- Nine fold ↑ Same as above

Table 2 contd...

6.	Cook and Monsen, 1977	In vivo	Semi-synthetic meal (4.1 mg)	Set I	Crystalline 0	1.29%	-
					25 mg (1.9)	2.42%	Nearly two fold ↑
					50 mg (3.8)	3.14%	Two and a half fold ↑
					100 mg (7.56)	5.12%	Four fold ↑
				Set II	0	1.53	-
					250 mg (18.9)	5.19%	Three fold ↑
					500 mg (37.8)	6.14	Four fold ↑
					1000 mg (75.6)	8.56%	Five and a half fold ↑
7.	Rossander et al 1979	In vivo	Wheat rolls, margarine, orange marmlade, cheese and coffee (3.1 mg)		Orange juice 70 mg (6.99)	3.7% to 8%	Two and a half fold ↑
8.	Hallberg and Rossander, 1982 (a)	In vivo	Rice, beans bread, margarine, apples, walnuts, almonds and yoghurt (5.8 mg)		Cauliflower 67 mg (3.58)	2.5% to 5.1%	Two fold ↑
9.	Hallberg and Rossander, 1982 (b)	In vivo	Hamburger string beans and potatoes (3.5 mg)		Orange juice 107 mg (9.5)	9.8% to 18.1%	Two fold ↑
10.	Hallberg and Rosander, 1984	In vivo	Maize, beans and rice (4.3 to 4.4 mg)		Crystalline 50 mg (3.5)	1.2% to 2.8%	Nearly two and a half fold ↑

Table 2 contd ...

11. Martinez-Torres et al, 1987	In vivo	Rice, maize bread and beans (3.9 mg)	Crystalline 15 mg (1.19) 30 mg (2.4)	6.3% to 6.9% to 21%	No significant increase Three fold ↑
12. Hallberg et al, 1989	In vivo	Wheat buns and margarine (4.1 mg)	Crystalline 0 50 mg (3.78)	22.4% 37.6%	One and a half fold ↑
13. Narasinga Rao and Prabhavati, 1978	In vitro available iorn #	Bajra (3.2 mg)	Crystalline 0 10 mg (0.96) 50 mg (4.8) 100 mg (9.7) 200 mg (19.4)	1.94% to 1.94% to 2.64% to 3.25% to 3.25%	No increase Slight increase Nearly two fold ↑ Same as above
14. Hazell and Johnson, 1987	In vitro	Wheat flour (3.3 mg)	Crystalline 250 mg (23.5)	4.0% to 7.7%	Nearly two fold ↑
15. Christian and Seshadri, 1989	In vitro available iron #	Wheat flour (6.4 mg)	Crystalline 0 100 mg (4.8) 200 mg (9.7) 300 mg (14.53) 600 mg (29.0)	3.74% 7.28% 8.09% 8.67% 9.84%	- Two fold ↑ Two fold ↑ Two and a half fold ↑ Nearly three fold ↑

In vitro available iron was calculated from % ionisable iron, using the prediction equation Y = 0.4707 x + 0.4827 where Y = % in vitro available iron and X = % ionisable iron (Narasinga Rao and Prabhavati, 1978).

1. Figures in parentheses represent molar ratio of ascorbic acid to iron

the ascorbic acid to iron molar ratio, higher the ratio, larger the magnitude of increase in absorption of iron from the basal value (Table 2 A).

Major findings in vitro: Three sets of in vitro studies have investigated the effect of ascorbic acid on iron availability from low availability meals. The iron content in these varied from 3.2 to 6.4 mg and the levels of ascorbic acid added ranged from 10 mg to 600 mg. The molar ratios of ascorbic acid to iron were 0.96 to 30. However, as shown in Table 2 A, the percent increase in iron availability with increasing molar ratios was of a much smaller magnitude in the in vitro system than in the in vivo system.

Dose effect : A well controlled human in vivo study by Cook and Monsen (1977) has clearly demonstrated that the increase in iron absorption was directly proportional (on a log-log scale) to the dose of ascorbic acid, upto 1000 mg level. The magnitude of increase brought about by the lower levels of ascorbic acid, however, was higher than that observed with the higher levels of ascorbic acid as shown in Figure 6. The other studies in the literature, summarised in Table 2, also demonstrate that the effect of ascorbic acid is dose related (Christian and Seshadri, 1989).

Interaction with other constituents : Ascorbic acid has been demonstrated to play a key role in counteracting the inhibitory effect of certain food constituents such as tannates and phytates

TABLE 2(A)

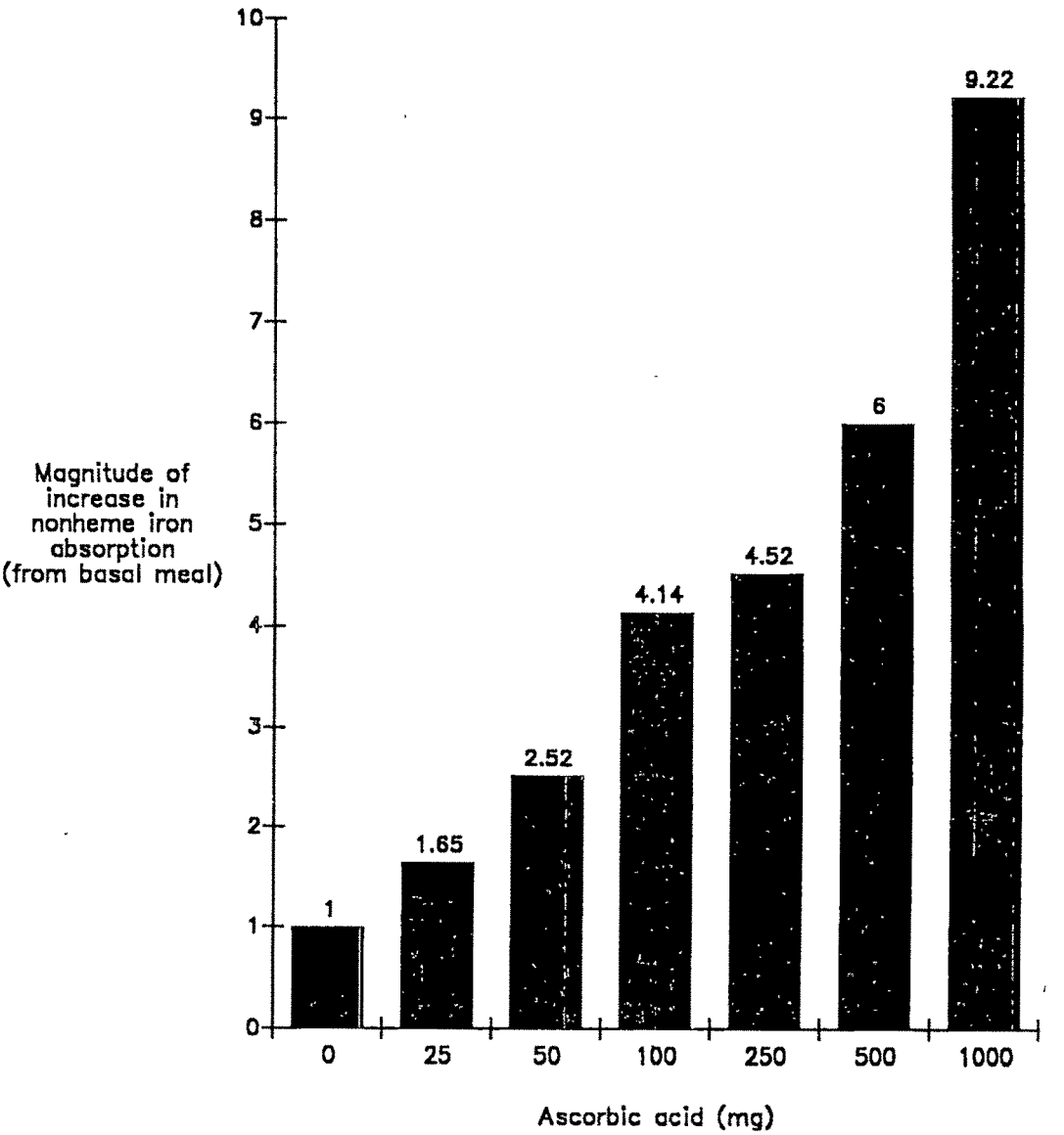
Effect of molar ration of ascorbic acid to iron on the magnitude of incereàse in iron absorption or availability in the in vivo and in vitro studies.

	Molar ratio of Ascorbic Acid to Iron (arranged in increasig order)	Magnitude of increase in iron absorption/availability (from the basal value, with no ascorbic acid)
In vivo		
	1.19	1.09
	1.76	1.50
	1.90	1.87
	2.40	3.30
	3.50	2.30
	3.58	2.04
	3.67	3.72
	3.78	2.43
	5.10	6.30
	5.40	5.72
	7.70	8.90
	15.50	9.31
In vitro		
I Set	0.96	0
	4.80	1.36
	9.70	1.67
	19.40	1.67
II Set	4.8	1.95
	9.7	2.16
	14.5	2.32
	29.0	2.63
III Set	23.5	1.92
	30.0	6.25

Figure 6

Magnitude of increase in absorption of nonheme iron when ascorbic acid was added to a 700 – kcal semi–synthetic meal; fed to healthy adult men, calculated as the ratio of with/without addition of ascorbic acid to the meal.

(Data taken from Cook and Monsen, 1977).



(Derman et al, 1977; Hallberg et al, 1989). From a meal of maize porridge and tea (195 mg tannin) addition of at least 250 to 500 mg ascorbic acid was required to bring about the same magnitude of increase (9 to 10 fold) in iron absorption as that observed with only 50 mg ascorbic acid, added to the maize meal without tea (Derman et al, 1977). In another study, it was indicated that about 80 mg ascorbic acid was required to fully counteract the inhibition brought about by 25 mg phytin phosphorus, from a wheat bun meal (Hallberg et al, 1989). The authors concluded, on the basis of their findings, that the addition of ascorbic acid was able to counteract, significantly, the inhibitory effect observed with phytate.

Effect of heating and storage : It has been reported by Hallberg et al (1982) that the enhancing effect of ascorbic acid on iron bioavailability can be significantly reduced if the food is held at warm temperatures (75^{o}C) for a period of 1 to 4 hours, especially in non-acidic conditions. This is explained by the fact that oxidation and heating destroys much of the ascorbic acid in food during cooking and subsequently holding at warm temperatures (Sayers et al, 1973). Hence it is imperative to consider the ascorbic acid content of the meals, as consumed, rather than that of the raw ingredients used, for intrepreting the enhancing effect on iron absorption of this food constituent.

It has been suggested that a meal that contains 100 mg net ascorbic acid either from native food sources or pure, crystalline form, can be classified as a high iron bioavailability meal (Monsen et al, 1978). Indeed such high

availability meals have been shown to have significant effect on hemoglobin regenertion in anemic humans.

Effect on Hb regeneration : Studies performed in our Department (Seshadri et al, 1985) have shown that supplements of 100 mg crystalline ascorbic acid, given daily along with lunch and dinner meals to anemic preschool children for a period of two months improved their Hb levels from 9.3 g/dl to 11.4 g/dl. This increase was similar to the increase brought about by prophylactic iron supplements (20 mg) (9.6 g/dl to 11.4 g/dl) given for the same period of time to anemic children.

Similar conclusion can be drawn from the findings of a recent study by Hunt et al, (1990) who investigated the effect of ascorbic acid on iron retention from a low iron bioavailability diet in 11 premenopausal women. The results of the study revealed that there was an increase in apparent iron absorption from 27 to 38% with 1500 mg ascorbic acid (in three divided doses) given daily for a period of 5.5 weeks. Ascorbic acid supplementation also improved the Hb, erythrocyte protoporphyrin and serum iron in these women.

A review of the studies pertaining to the effect of ascorbic acid on iron availability demonstrates that addition of ascorbic acid improves nonheme iron absorption, and also brings about an improvement in Hb levels, especially in anemic subjects. However, the question whether consumption of ascorbic acid will lead to increased stores of iron as measured by serum ferritin has been disputed by Cook et al (1984). They have reported that

vitamin C supplements (2 g), given daily with meals to 17 normal adult subjects for 4 months did not improve their serum ferritin status (46 mg and 43 mg/l before and after supplementation respectively). Continuing the supplements for an additional 20 months period also failed to show any improvement in serum ferritin. This indicated that ascorbic acid supplements did not have any appreciable enhancing effect on iron reserves of the subjects, as measured by serum ferritin. The authors concluded, therefore, that imporoving the bioavailability of dietary nonheme iron did not result in any improvement in the iron stores of the individuals. It may be noted however, that the individuals in this study were already consuming diets which contained appreciable amount of meat, which could have promoted nonheme iron absorption, minimizing any further effect of supplemental ascorbic acid on iron status of the subjects. Studies of Smith and Bidlack (1980) are of great interest in this context. They investigated the interrelationship of dietary ascobic acid and iron on the tissue distribution of ascorbic acid, iron and copper in female guinea pigs. It was shown that high dietary ascorbic acid (225 mg vs 25 mg in control) increased tissue ascorbic acid levels and produced a concomitant decrease in liver ferritin and hemosiderin while the total iron in the spleen was increased. The authors suggested, as an explanation for this, that ascorbic acid probably facilitated the reduction of ferric iron (from liver ferritin and hemosiderin) to ferrous form and possibly increased the enzymic incorporation of iron into porphyrin thereby increasing the heme iron pool in the spleen. It seems,

therefore that ascorbic acid may be involved in mobilising iron stores resulting in greater biosynthesis of heme iron in the body.

Mechanism of action : Ascorbic acid operates in multiple ways to facilitate iron absorption. In a classical study by Conrad and Schade (1968), it was demonstrated that ascorbic acid formed a soluble chelate with $FeCl_3$ at an acid pH, (pH2) which remained soluble even when the pH was increased to alkalinity (pH12). The authors explained that there could be two types of chelates formed, depending upon the pH of the medium in which the reaction is initiated between ascorbic acid and the ferric iron.

$FeCl_3$ + ascorbic acid + base -> soluble iron chelate (No.1)
(at acidic pH)

$FeCl_3$ + base + ascorbic acid -> insoluble iron chelate (No.2)
(at alkaline pH)

The authors suggested that the ability of ascorbic acid to combine with food iron at a lower pH in the stomach results in an iron ascorbic acid complex (no.1) which remains stable and soluble even as the pH increases in the intestine. In this manner, ascorbic acid also prevents the complexing of iron with other inhibitory ligands which react at the alkaline pH of the intestine to make it insoluble (Hurrel, 1984).

Another mode of action of ascorbic acid is through its strong reducing action on ferric iron converting it to a more soluble ferrous iron. It has been shown by Greenberg et al (1957) that presence of strong reducing agents and anti-oxidants

such as ascorbic acid and tocopherol in the diet along with iron resulted in greater Hb regeneration in anemic rats as compared to the controls. The authors could not pinpoint, though, whether the improved Hb picture of the experimental group receiving iron along with the two vitamins was due to an increased absorption of iron, an increased rate of utilization of iron or both.

It has also been suggested that ascorbic acid may preferentially donate its iron to the brush border receptors at the mucosal lining, thereby increasing its uptake by the mucosal cells (Lynch, 1980).

Animal tissue

Animal tissue contributes highly available heme iron in the diet. However, a more important contribution of heme iron is its property to enhance the absorption of nonheme iron contained in the same meal.

Layrisse et al (1968) were the first to report this enhancing effect of animal protein on absorption of nonheme iron. In a subsequent study, they confirmed this observation (Martinez-Torres and Layrisse, 1970) by reporting that addition of 100 g fish brought about a two fold increase in iron absorption from black beans.

A number of studies later reconfirmed the phenomenon of enhancing effect of animal tissue on iron absorption, using a variety of animal products such as beef, lamb, chicken, pork,

fish, liver, or calf thymus tissue (Cook and Monsen, 1976; Bjorn-Rasmussen and Hallberg, 1979). The potency of 100 g cooked meat in enhancing iron absorption has been graded as equal to that of 100 mg ascorbic acid (Monsen et al 1978; Monsen and Balintfy 1982).

The exact nature of the enhancing factor(s) present in animal protein is not known. However, it is speculated that the amino acids and/or the polypetides arising from proteolytic digestion might chelate iron, thereby facilitating its absorption (Hallberg and Bjorn Rasmussen, 1972; Morck and Cook, 1981). Some evidence in support of this hypothesis comes from the study of Layrisse et al (1984) who observed that reduced glutathione, a tripeptide, increased significantly the absorption of nonheme iron present in black beans and corn. The same authors in a subsequent study have reported that cysteine containing peptides rather than the free amino acids are responsible for enhancing intestinal iron absorption in man from a maize meal (Taylor et al, 1986). It has been suggested that these samll peptides may improve the solubility of iron by forming tridentate iron chelates (Hazell, 1985). It is also possible that cysteine and glutathione, by their reducing action, may convert ferric to ferrous ion, thereby increasing its solubility at the alkaline pH of the intestine. It has been argued, however, by Bjorn Rasmussen and Hallberg (1979) that amino acid composition of meat and fish (regarded as enhancers) is very similar to that of eggalbumen which is supposed to be inhibitory in nature. They suggest therefore, that there is possibly a 'meat factor', which

is not present in egg, cheese and such nontissue animal proteins, which is responsible for an enhancing influence of meat, fish and poultry on nonheme iron absorption.

Citric acid

Citrus fruits contain high concentrations of citric acid, which has been identified as a potential enhancer of iron availability (Hazell, 1985). There are only a few studies which have investigated the effect of citric acid on nonheme iron absorption. These are summarised in Table 3.

Major findings in vivo : As is evident from the Table, the iron content of the meals that have been used in the in vivo studies outlined above, lay within a narrow range of 3 to 4.3 mg and the level of citric acid tested ranged from a low of 36 mg to a high dose of 4700 mg.

Of the three in vivo studies, two reported an enhancing effect of citrate on iron absorption (Gillooly et al, 1983; Ballot et al, 1987), while the third one actually reported a reduction in iron absorption on addition of 1 g of citrate (Hallberg and Rossander, 1984). The reasons for such an inconsistent finding of the third study are obscure. Of the studies indicating an enhancing effect of citrate, addition of 1 g citrate per se, to a rice meal was reported to result in a three fold increase in iron absorption. On the other hand, a combination of citrate (750 mg) and ascorbic acid (33 mg) added in crystalline form or as orange juice brought about 5 to 7 fold

TABLE 3

Effect of citrate on iron absorption/availability in the in vivo and the in vitro systems

	Reference	Type of study	Basal meal and iron content	Form and quantity of citrate	Presence of other factors	Effect on iron absorption	Direction and quantum of increase
1.	Gillooly et al, 1983	In vivo	Rice meal (3 mg)	Crystalline		GM*	-
				0	0	0.049 mg	
				36 mg	0	0.099 mg	Two fold ↑
			Rice meal (3 mg)	0	0	0.028 mg	-
				1000 mg	0	0.085 mg	Three fold ↑
2.	Hallberg and Rossander 1984	In vivo	Maize, rice and black beans (4.3 mg)	Crystalline			
				0	0	2%	
				1000 mg		0.8%	nearly one third ↓
3.	Ballot et al, 1987	In vivo	Rice meal (3 mg)	Crystalline		GM*	-
				0		0.025 mg	four and a half fold ↑
				0	33 mg ascorbic acid	0.114 mg	
				750 mg	33 mg ascorbic acid	0.17 mg	nearly seven fold ↑
				0	28 mg ascorbic acid	0.098 mg	four fold ↑
				Fresh orange juice 750 mg	28 mg ascorbic acid	0.139 mg	five and a half fold ↑
				Commerical orange juice 700 mg	30 mg ascorbic acid	0.166 mg	six and a half fold ↑
				Lab. Lemon juice 4700 mg	30 mg ascorbic acid	0.226 mg	nine fold ↑

Table 3 contd...

4.	Kojima et al, 1981	In vitro solubilization of iron	Beans	0	0	13%	-
				1 mM	0	15%	Slight increase
				5 mM	0	22%	1 1/2 times ↑
				10 mM	0	27%	Two fold ↑
				15 mM	0	29%	Same as above
				20 mM	0	38%	three fold ↑
				Fresh orange juice (7 mM citrate)	3 to 8 mM ascorbic acid	24% to 68%	nearly three fold ↑
5.	Hazell and Johnson, 1987	In vitro	Wheat flour (3.2 mg)	Crystalline 0	0	3.8%	-
				627 mg	0	19%	Five fold ↑
			100 g digest containing 10g wheat flour	Fresh orange juice 325 mg	32 mg ascorbic acid	25%	Six fold ↑
				Heated, stored orange juice 303 mg	0	17%	Four fold ↑
				crystalline 303 mg	0	16.7%	same as above
				0	25 mg ascorbic acid	7.7%	two fold ↑
				crystalline 450 mg	25 mg ascorbic acid	26.8%	six and a half fold ↑

* Geometric mean

enhancement in iron absorption from the same meal (Ballot et al, 1987). This indicated that citrate and ascorbate together exerted a much more potent enhancing effect than either of them present alone in the meal.

Major findings in vitro : The two in vitro studies that have investigated the effect of citrate have both shown an enhancement of iron availability, on addition of citrate, either in crystalline from or as orange juice. Addition of citrate per se (627 mg) brought about a five fold increase in iron availability (Hazell and Johnson, 1987), while addition of fresh orange juice containing both citrate and ascorbate resulted in a greater magnitude of enhancement (six to eight fold) (Kojima et al, 1981; Hazell and Johnson, 1987). This trend is essentially similar to that observed in the in vivo situation.

Dose effect : Addition of increasing doses of citrate (from 1 mM to 20 mM) resulted in a very gradual increase in total soluble iron while the Fe^{++} fraction remianed constant throughout the concentrations of citrate, being approximately 1/4th of the total soluble iron at each level of addition (Kojima et al, 1981).

Effect of heating : An interesting and encouraging finding, reported by Hazell and Johnson (1987) was that orange juice, whose ascorbate content was completely destroyed by heating and storage, could still produce an appreciable enhancement in the in vitro iron availability. The authors suggested that since citrate was not affected by heating and storage, it seemed to be

a far more effective enhancer than ascorbic acid, in improving the availability of iron from cereal foods.

Mechanism of action : The mechanism by which citrate helps to enhance iron availability is not very clear. It has been hypothesised that citrate which has three carboxylic groups and a hydroxyl group, forms loosely bound chelate with iron at the neutral or weakly alkaline pH, thereby preventing polymerization of iron hydroxides at that pH (Hazell, 1985). This unstable citrate iron chelate remains soluble in the intestinal lumen and hence helps in a higher uptake of iron. This was experimentally demonstrated by Kojima et al (1981) in the in vitro system where citrate was shown to be maximally effective in mobilizing iron from beans near pH 6 and not at acidic pH (pH2). When ascorbic acid was present concomitantly, there was greater enhancement of iron availability. The authors suggested that this could be because ascorbic acid, being a reductant reduces ferric form of iron to ferrous form and influences iron mobilisation under acidic conditions. Citrate on the other hand forms soluble chelates with iron at the alkaline pH of the intestine. Hence together they promote iron availability in a co-operative manner, resulting in an additive enhancing effect on iron availability (Kojima et al, 1981).

Studies by Spiro et al (1967a) have examined the chemistry of the iron-citrate reactions and have revealed that at low pH (less than 4) citrate binds with ferric form of iron in equimolar ratios, resulting in an anionic chelate formation (FeCit-). On subsequent increase in the pH, this chelate probably results in

formation of low molecular weight polymer $\{(FeCit)_2 OH\}_n^3$ which is readily dialyzable. It has been suggested by the authors, that the coating of citrate on the citrate - iron chelate, is possibly responsible for preventing the polymer to precipitate at high pH. If excess of citrate is present in the solution, it helps in the formation of another compound $\{Fe(Cit)_2\}^{5-}$ which is more soluble than the polymer $\{(FeCit)_2 O_4\}^{3-}$. Also, the formation of $\{Fe(Cit)_2\}^{5-}$ is carried out at the expense of the polymerisation reaction, thereby helping in greater total solubility of ferric iron at the intestinal pH (Spiro et al, 1967b). It has also been hypothesised that citrate, when present in excess may bind with certain precipitating salts such as calcium, thereby preventing their complex formation with iron (Hazell and Johnson, 1987).

Inhibitors

In general, nonheme iron absorption in man is low and the presence of inhibitors of iron absorption further reduces the rate of nonheme iron absorption from complex meals that contain these factors in larger proportion than the enhancers. These include certain chemical groups and ligands such as tannates (in tea), phytates (in bran), oxalates and calcium and phosphate salts which are reported to form insoluble complexes and macromolecules with iron resulting in its poor availability (Morck and Cook, 1981).

Tannates (and polyphenols)

The early indication that tannates and polyphenols might be inhibitory in nature came from the studies of Disler et al (1975) who showed that a cup of tea drunk with a bread meal reduced iron absorption from 11.5 to 2.5%. Similarly, the mean absorption of iron from a maize meal was reported to be reduced from 3.8 to 2.1% when tea was drunk with it (Derman et al, 1977). The factor responsible for such an inhibitory effect of tea on iron absorption was identified to be tannin.

Studies that have investigated the effect of tannins and other polyphenols in pure chemical form or native food form in the in vivo and in vitro system, on iron availability are summarised in Table 4.

Major findings in vivo : The meals tested in vivo contained 2.0 to 3.8 mg of iron while the level of tannins/polyphenols added varied from a low of 5 mg to a high of 500 mg. The results of these in vivo studies reveal that presence of tannins (or polyphenols), in a pure chemical form or in a natural food form, markedly reduces iron absorption. In general, addition of tea to a meal resulted in 46 to 71% reduction in iron absorption (Derman et al, 1977; Rossander et al, 1979; Hallberg and Rossander, 1982 and Brune et al, 1989), while addition of pure tannic acid in varying amounts (5 to 500 mg) resulted in a proportional decrease in iron absorption from 24 to 95% (Gillooly et al, 1983; Brune et al, 1989). Food products rich in polyphenols such as spinach and oreyano were also reported to inhibit iron absorption in

TABLE 4

Effect of tannins (and polyphenols) on iron absorption/availability in the in vivo and the in vitro systems

	Reference	Type of study	Basal meal and iron content	Form and quantity of tannins (polyphenols)	Effect on iron absorption	Quantum of reduction
1.	Derman et al, 1977	In vivo	Maize porridge 2 mg	No tea	3.8%	44.7% ↓
				Tea	2.1%	
2.	Rossander et al, 1979	In vivo	Wheat rolls margarine, cheese marmalade and coffee 2.8 mg	No tea	5.7%	
				Tea	2.5%	56% ↓
3.	Hallberg and Rossander, 1982	In vivo	Hamburger string beans and potatoes 3.0 mg	No tea	10.8%	62% ↓
				Tea	4.1%	
4.	Morck et al, 1983	In vivo	Hamburger meal	Coffee	-	39% ↓
				Tea	-	64% ↓
			Semi-purified meal	-	5.88%	-
				Drip coffee	1.64%	72% ↓
				Instant cofee	0.97%	83% ↓
				Double strength of instant coffee	0.53%	98% ↓
5.	Gillooly et al, 1983	In vivo	Broccoli	Crystalline tannic acid 500 mg	29.7% (Basal) to 1.5%	95% ↓

Table 4 contd...

6.	Brune et al, 1989	In vivo	Wheat rolls and margarine 3.8 mg	Crystalline tannic acid 5 mg	31.2% (Basal) to 23.6%	24% ↓
				10 mg	24.8% (Basal) to 15.2%	39% ↓
				25 mg	28.8% (Basal) to 9.6%	67% ↓
				50 mg	23.6% (Basal) to 3.2%	86% ↓
				100 mg	24.8% (Basal) to 2.8%	89% ↓
				200 mg	20.8% (Basal) to 1.6%	94% ↓
				Gallic acid 14.7 mg	20.8% (Basal) to 10.4%	50% ↓
				Chlorogenic acid 30.5 mg	16.8% (Basal) to 11.6%	31% ↓
			with oregano 5.1 mg	Catechin 161 mg + tannic acid 32 mg	20.8% (Basal) to 6.8%	67% ↓
			with spinach 4.8 mg	Catechin 4 mg + tannic acid 21 mg	28% (Basal) to 19.6%	30% ↓
			with coffee 3.8 mg	Catechin 162 mg + tannic acid 12 mg	16.8% (Basal) to 6.4%	62% ↓
			with tea 3.8 mg	Catechin 15 mg + tannic acid 27 mg	20.4% (Basal) to 6.0%	68% ↓

Table 4 contd . . .

7.	Narasinga Rao and Prabhavati, 1978	In vitro	Jaggery 4.6 mg	Crystalline tannic acid 0	8%	-
				50 mg	6.7%	16% ↓
				100 mg	4.7%	41% ↓
				200 mg	3.7%	53% ↓
8.	Narasinga Rao and Prabhavati, 1982	In vitro	Redgram dhal 4.1 mg	Crystalline Tannic acid (mg & molar ratio to iron) 0	5.47%(Basal)	
				upto 38 mg (10:3)	5.47%	No reduction
				64 mg (10:5)	4.1%	24% ↓
				127 mg (10:10)	2.7%	50% ↓
				225 mg (10:20)	2.2%	58% ↓
				382 mg (10:30)	0%	100% ↓
			Wheat 6.9 mg	0	2.5%	Basal
				upto 62 mg	2.5%	No reduction
				125 mg (10:6)	1.8%	26% ↓
				377 mg (10:18)	1.1%	54% ↓
				628 mg (10:30)	0%	100% ↓
			Whole Bengalgram 6.7 mg	Tannin content 330 mg	1.7%	-
			Whole Greengram 7.6 mg	Tannin content 560 mg	1.3%	-
			Whole Redgram 3.7 mg	871 mg	0%	-

Table 4 contd . . .

8.	Narasinga Rao and Prabhavati, 1982	In vitro	Breakfast meal I 13.5 mg	Tea (195 mg/cup)	2.7% to 1.87% (Basal)	33% ↓
			Breakfast II 11 mg	Tea (195 mg/cup)	2.5% to 1.76% (Basal	30% ↓
				Tamarind extract added to various meals/dals (600 mg tannin/10 g tamarind)		Nearly 70% ↓ in ionisable iron
9.	Rao and Daosthale, 1988	In vitro	Brown Ragi 4.9 mg	Tannin content 914 mg	-	Removal of tannin from the grain increased ionisable iron content by 85%
			White Ragi 5.8 mg	Tannin extract from brown ragi	-	Reduced ionisable iron by 52 to 65%
10.	Christian and Seshadri, 1989.	In vitro	Cereal meal (wheat) 6.4 mg	No tea	3.92%	-
				tea (195 mg)	2.56%	35% ↓

In vitro available iron was calculated from % ionisable iron, using the prediction equation Y= 0.4707 x + 0.4827 where Y = % in vitro available iron and x = % ionisable iron (Narasinga Rao and Prabhavati, 1978)

proportion to their respective phenolic groups (Brune et al, 1989). Further, it has been reported by Gillooly et al (1983) that certain vegetables that contained high polyphenol content, exhibited low iron absorption values as compared to the vegetables that had low or moderate content of polyphenols.

Major findings in vitro : Though the findings of the in vitro system are in the same direction as those observed in the in vivo system with respect to the inhibitory effect of tannins (and polyphenols) on iron availability, there are some differences in the magnitude of decrease brought about by tannins in the in vitro situation. Addition of tea (1 cup; 195 mg tannin) to a meal in the in vitro system brought about 30 to 33% reduction in iron availability (Narasinga Rao and Prabhavati, 1982), while in the in vivo system, tea was reported to decrease iron absorption from 46 to 71%. Similarly, addition of 100 mg pure tannic acid to a meal brought about 26 to 50% reduction in in vitro iron availability, vis-a-vis nearly 90% decrease in iron absorption on addition of the same amount of tannic acid in the in vivo system (Narasinga Rao and Prabhavati, 1978 and 1982; Brune et al; 1989 respectively). This difference in the magnitude of inhibition brought about by the same quantities of tannate may be explained by the observation that in the in vitro system, nearly three moles of tannic acid are required to bind one mole of iron (Narasinga Rao and Prabhavati, 1982), while in the case of in vivo system, only 0.5 mole of tannic acid is required to bind a mole of iron (Brune et al, 1989). Therefore, in order to bring about the same magnitude of inhibition in the in vitro situation, as observed in

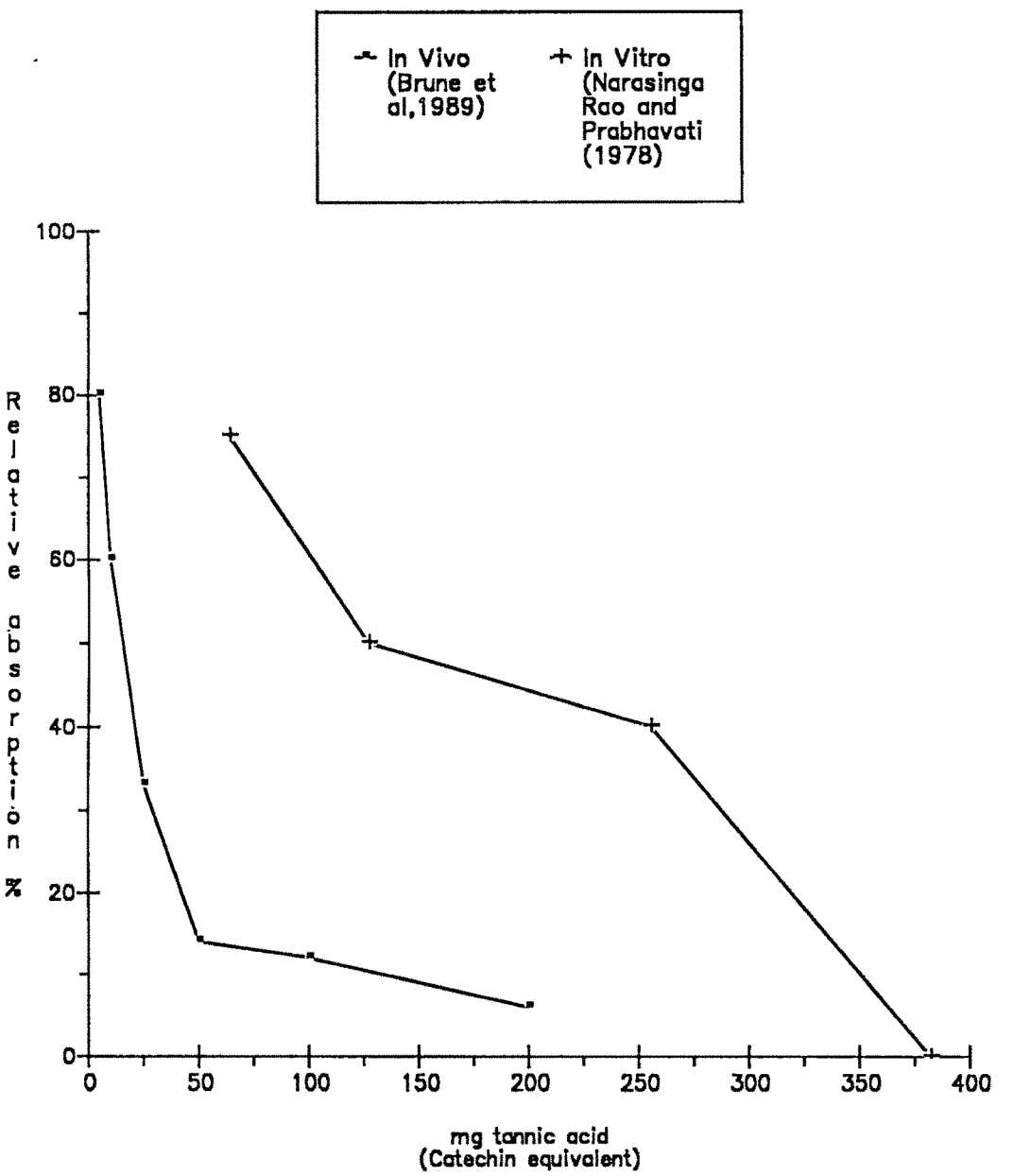
the in vivo situation, higher quantities of tannate would be required. Nonetheless, in both the systems, a progressive fall in iron availability is observed on addition of increasing levels of tannic acid to the meal or foods containing varying amounts of polyphenols.

Dose effect of tannin : Effect of increasing dose levels of pure tannic acid on iron availability has been studied in both in vivo and in vitro situation (Brune et al, 1989; Narasinga Rao and Prabhavati, 1982). The results of these studies are shown in Table 4 and Figure 7. It can be seen that when tannic acid was added to foods that do not normally contain any native tannin, there was a progressive fall in iron availability until the iron content of the diet was almost completely bound with tannin, resulting in 95 to 100% reduction in iron availability values. In other words, the inhibitory effect of tannins was strongly dose related.

Mechanism of action : Structurally, tannins are divided into two sub-groups, condensed tannins which are polymers of flavanols (e.g catechin) and hydrolysable tannins which contain gallic acid. The reactive potential of the phenolic molecule lies in the hydroxyl groups, which differ from compound to compound. Catechin containing polymers have two hydroxyls attached to the aromatic ring while the galloyl group of compounds contain three hydroxyles attached to the ring (Figure 8). It has been found that these substances readily complex with iron to form a blackish precipitate (Brune et al, 1989). This is because the

Figure 7

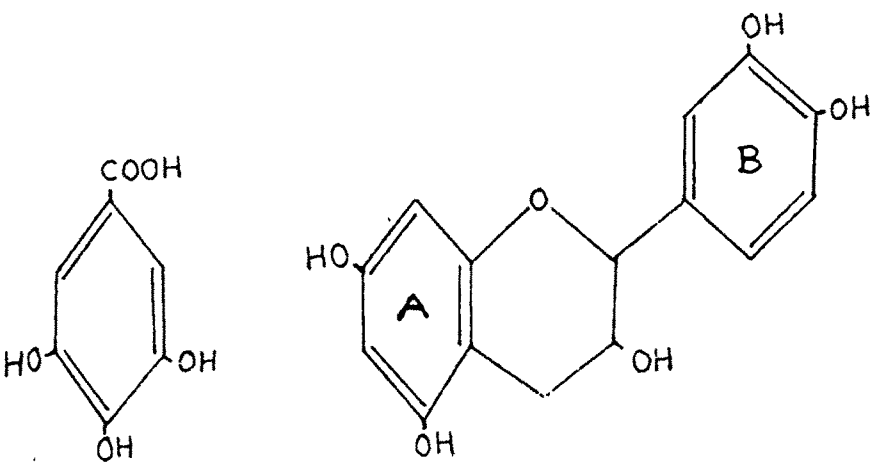
Dose effect of tannin on relative absorption of iron, calculated as the ratio of with to without addition of tannin to the meal.



hydroxyl groups present in the aromatic ring of polyphenols react with iron, resulting in formation of insoluble complexes at the intestinal pH, thus making the iron unavailable for absorption.

Figure 8

Structural formulae of (a) gallic acid and (b) Catechin. (Reproduced from Brune et al, 1989)



When Brune et al, (1989) studied the effect of catechol and galloyl group of polyphenols on iron absorption, it was observed that gallic acid inhibited iron absorption to the same extent as tannic acid while catechin did not show any inhibition. These results suggested that the group of condensed tannins was less interfering with iron absorption than the hydrolysable tannins. The authors concluded therefore, that the galloyl group of phenolic compounds, may be responsible, mainly, for the inhibition of iron absorption from foods and beverages containing these substances. The inhibition of iron absorption is approximately in proportion to the respective content of galloyl groups in these foods. The group of condensed tannins, on the other hand is of minor importance.

Phytates (and bran)

Widdowson and McCance (1942), in a classical balance study on humans, indicated that less iron was absorbed from brown bread as compared to white bread. High content of bran in the brown bread was thought to be responsible for such an inhibitory effect on iron absorption. Later, this effect of bran was confirmed by several other studies, using more recent, radioiron techniques for determining iron absorption (Bjorn-Rasmussen, 1974; Morris et al, 1980; Simpson et al, 1981; Hallberg et al, 1987). A quantitative relationship between the amount of bran added to a meal and the reduction in iron absorption was also demonstrated using radioiron technique (Bjorn-Rasmussen, 1974). The factor,

responsible for the inhibitory effect of bran was thought to be phytate. Studies that have investigated the effect of bran or phytate in modifying the availability of iron from diets are summarised in Table 5.

Major findings in vivo : While the inhibitory effect of bran has been well documented in most of the studies, effect of pure crystalline phytate in influencing iron absorption is reported to be contradictory in nature. On the one hand, animal studies, using pure Na-phytate or monoferric phytate have shown no inhibitory effect (Gordon and Chao, 1984) of adding this food constituent to animal feed. On the other hand, several in vivo human studies have reported a significant reduction in iron absorption on addition of Na-phytate, mono ferric phytate or Mg-K-phytate (Gillooly et al, 1983; Hallberg et al, 1989). However, some in vivo human studies have indicated that the inhibitory effect of bran or soy could not be explained by the high phytate content of these foods (Simpson et al, 1981; Hallberg and Rossander, 1982; Hurrel et al, 1988). These observations suggest that the effect of pure phytate may not be representative of its action when it is present endogenously in foods.

Major findings in vitro : There are only a couple of in vitro studies available, that have investigated the effect of phytate on iron availability (Narasinga Rao and Prabhavati, 1978; Hazell and Johnson, 1987). Both these studies have indicated a depressing effect of Na-phytate. One of the studies however suggested that the level of intake of phytate in an average UK

TABLE 5

Effect of phytate on iron absorption/availability in the in vivo and in vitro systems

	Reference	Technique used	Form and Quantity of phytate	Effect on iron absorption/ availability	Direction of effect
1.	Cowan et al, 1966	In vivo Rat model (Hb regeneration)	45 to 75% phosphorus in the diet replaced by phytin-P	No significant effect on iron absorption	Neutral (no inhibition)
2.	Morris and Ellis, 1976	In vivo Rat model (RBV)	Monoferric-phytate (M-F-Phy)	RBV of M-F-Phy was equal to that of the reference compound ferrous-ammonium sulfate	Neutral (no inhibition)
3.	Lipschitz et al, 1979	In vivo Dog model	M-F-Phy	M-F-Phy was in soluble state and available for absorption	Neutral (no inhibition)
4.	Ellis et al, 1982	In vivo Rat model (Hb response)	Ferric-phytate	Same Hb-reponse for ferric-phytate and ferrous ammonium sulfate	Neutral (no inhibition)
			Ca-Fe-Phy	Ca_3Fe-Phy was less available than both ferric-phy or Ferrous Amm. Sulfate	Inhibition by Ca-Fe-Phy complex
5.	Gordon and Chao, 1984	In vivo Rat model (Hb regeneration)	Na-Phytate (0.66%)	Significantly increased RBV of the ferrous iron from 100 to 124%	Enhancing effect

Table 5 contd . . .

6.	Chausow and Czarmeck, 1988	In vivo Chick model (RBV)	Sesame seed meal	RBV of 69% as compared to 100% from $FeSo_4$	Neutral (No inhibition)
			Rice bran	RBV of 77%	Slight inhibition
			Soyabean meal	RBV of 45%	Significant inhibition
7.	Simpson et al, 1981	In vivo Human study			
			Wheat bran (12 g per meal)	51 to 74% reduction in iron absorption	Inhibitory effect (of bran)
			M-F-Phy (labelled)	absorbed as well as the common pool of dietary iron	Neutral (inhibition was not explained by M-F-Phy)
			Dephytinising the bran by endogenous phytate	as inhibitory as the bran	
			Soluble fraction of dephytinised bran (rich in phosphate)	inhibitory in nature	Inhibitory effect of phosphate
			Insoluble, high fibre fraction	No inhibition	Neutral
8.	Hallberg and Rossander, 1982	In vivo Human study	Removal of phytate from soy flour by acid washing	No improvement in iron absorption	Neutral (inhibitory effect of soy flour is not explained by its phytate content alone)
9.	Gillooly et al, 1983	In vivo Human study	Na-phytate (2 g)	GM* from 0.152 mg (basal meal) to 0.035 mg (with phytate)	Inhibition
10.	Gillooly et al, 1984	In vivo Human study	Sorghum perlings rich in phytate	GM* 0.015 mg as compared to 0.035 mg absorption from the pearled albino sorghum	Inhibition

Table 5 contd . . .

10. Gillooly et al, 1984	In vivo Human study	Na-phytate	GM* 0.185 (basal meal) to 0.037 (with phytate)	Inhibition
		Wheat bran	Significant reduction in iron absorption	Inhibition
11. Hallberg et al, 1987	In vivo Human study	Dephytinised wheat bran (with acid)	Removal of inhibitory factors, present in bran	-
		Reconstitution with Na-phytate	Inhibition comparable to that with bran	Inhibition
		Dephytinised bran (with endogenous phytase)	Removal of almost all the inhibition observed with bran	-
		K-Mg-phytate	Inhibitory effect, compareable to that with bran	Inhibition
12. Hurrel et al, 1988	In vivo Human study	Removal of 90% phytate from soy-protein isolate	No improvement in iron absorption	Neutral (no inhibition)
13. Hallberg et al, 1989	In vivo Human study	Na-phytate (2 mg to 250 mg)	2 mg - 18% 25 mg - 64% 250 mg- 82%	Dose dependent inhibition
14. Narasinga Rao and Prabhavati, 1978	In vitro study	Na-phytate 6 mg 12 mg 24 mg	9.6% to 7.4% } 22% (Basal)to 5.4% } to 70% to 2.8% }	Inhibition
15. Hazell and Johnson, 1987	In vitro study	Na-phytate (234 mg) per 100 g wheat flour	approx. 3.6% } 72% (Basal) } to 1% }	Inhibition

* Geometric mean

diet (80 mg/person/day) would not possibly result in any significant depression in iron availability (Hazell and Johnson, 1987).

Dose effect : Very recently, a strong dose dependent reduction in the in vivo iron absorption has been reported by Hallberg et al (1989) on addition of increasing doses of pure Na-phytate. The authors found that addition of as low as 2 mg Na-phytate to a wheat roll and margarine diet resulted in 18% reduction in iron absorption. As the amount of phytate was increased, there was a proportional decrease in iron absorption values, being 64% reduction with 25 mg phytate and 82% decrease with 250 mg phytate. The magnitude of reduction with phytate was, however, more marked in the lower dose level range (2 to 10 mg) than in the higher dose level range (25 to 250 mg).

Interaction with other constituents : Hallberg et al (1989) have investigated if ascorbic and/or meat can counteract the inhibitory effect of pure Na-phytate in the in vivo human system. Their findings revealed that 50 mg ascorbic acid was not sufficient to completely counteract the inhibitory effect of 25 mg phytin phosphorus. Increasing the level of ascorbic acid to 80 mg could however, counteract the effect of phytate (25mg). Fifty g of meat was not strong enough to reverse the reducing effect of 25 mg phytin phosphorus, though it is known to be a strong enhancer of iron absorption.

Effect on Hb regeneration : The in vivo studies, using various animal models, to investigate the effect of phytate/monoferric phytate/bran on Hb regeneration have unanimously reported no inhibitory effect of this food constituent on iron absorption. An interesting finding is that in a rat model, Na-Phytate is even shown to be enhancing in nature (Gordon and Chao, 1984). Phytate, in its native food form also (sesame seed meal) shows results comparable to those observed with a highly available reference compound, ferrous sulfate, in chicks (Chausow and Czarmeck, 1988).

Mechanism of action : Recently Graf (1983) has proposed a hypothesis that may explain, partly, the conflicting role of phytate in influencing iron absorption. According to him, 'all metal-phytate complexes at a low metal to phytate ratio may be soulble (and hence bioavailable) at the intestinal pH'. Hence, monoferric phytate which is highly soluble may have its iron well absorbed (Morris and Ellis, 1976). However, when ohter mineral cations are also present in the intestinal lumen, such as calcium ions, formation of calcium-iron-phytate (Ca_3Fe phytate) may take place which is relatively insoluble and its iron is poorly absorbed (Ellis et al, 1982; Platt and Clydesdale, 1987). These studies indicate that presence of other ligands in the lumen may have a strong influence on the resultant availability of iron, in the presence of phytate. The possible factors include calcium, phosphorus, tannins and other polyphenolic compounds. It is also proposed that a high fibre content of bran and other food products, may, in association with phytate, be responsible for

reducing iron bioavailability in humans (Morck and Cook, 1981; Morris, 1983).

Oxalate

Available literature on the effect of oxalate on iron absorption is fragmentary and contradictory, as shown in Table 6.

Major findings in vivo : The in vivo studies that have investigated the effect of spinach or pure oxalate have reported varying findings. The in vivo human study of Gillooly et al (1983) revealed a moderate inhibitory effect of oxalate (1 g calcium oxalate) on iron absorption from cabbage. However, the authors observed that the iron absorption values for certain vegetables varied widely even though they all contained high levels of oxalate. They suggested, therefore that bioavailability of iron from these vegatables may be dependent upon the presence of other inhibitors and enhancers, rather than the presence of oxalate per se.

When effect of spinach was studied by other investigators using a rat model, either there was no inhibition (Van Campen and Welch, 1980) or only moderate inhibition (Gordon and Chao, 1984). In order to pinpoint the factors responsible for the inhibitory effect of spinach in the latter study, the authors compared the relative biological value in rats, of pure crystalline oxalate (2.1%), oxalate + cellulose (3.75%) and spinach in the diet, taking FeSo4 as the reference standard (RBV = 100%). It was observed that oxalate per se or oxalate + cellulose in the diet

TABLE 6

Effect of oxalate on iron absorption/availability in the in vivo and in the vitro systems

	Reference	Type of study	Form/Quantity of oxalate	Effect on iron absorption/ availability	Direction of effect
1.	Van Campen and Welch 1980	In vivo Rat model	Spinach (2.1% oxalate)	RBV of spinach was comparable to that from $FeCl_3$ or iron-oxalate	Neutral (No inhibition)
2.	Gordon and Chao, 1984	In vivo Rat model	Spinach in diet	53% RBV against 100% from $FeSO_4$ diet	Inhibition (by spinach)
			Oxalate (2.1%)	164% RBV	Enhancing effect of oxalate
			Oxalate + Cellulose (3.75%)	138% RBV	Enhancing effect (though less than oxalate per se)
3.	Gillooly et al, 1983	In vivo Human study	Calcium oxalate (1 g) added to cabbage	GM* from 0.32 mg (basal to 0.195 mg	Inhibition
4.	Kojima et al, 1981	In vitro	Spinach (added to beans)		
			Supernatant	7% ↓ in solubilization of iron	Neutral (No significant inhibition)
			Precipitate	50% ↓ in solubilization of iron	Inhibition (by spinach)
			Whole suspension	80% ↓ in solubilization of iron	
5.	Shah, 1983	In vitro	Cereal meals without spinach	2.8% iron availability	
			Meals with spinach	1.2% iron availability	Inhibition (by spinach)
6.	Chawla et al, 1988	In vitro	Green leafy vegetables (GLVs)	Trend of decreased iron availability with very large amounts of oxalate in GLVs	No significant correlation

* Geometric mean

produced a significant enhancement in iron absorption as reflected by RBVs of 164% and 138% respectively. Spinach on the other hand, showed inhibition (RBV = 35%),indicating that there may be some other factors, besides oxalate and cellulose, which may possibly be responsible for such an inhibitoy effect of spinach on iron absorption.

Major findings in vitro : The in vitro study of Kojima et al (1981) where spinach was reported to be inhibitory in nature, suggested that since spinach is rich in oxalate and fibre, these factors may explain, partially, the decrease in iron availability observed with this green leafy vegetable (GLV). Studies from our department have also observed an inhibitory effect of certain GLVs on in vitro iron availability (Shah, 1983; Chawla *et al*, 1988). However, no direct evidence is available to suggest that it is the oxalate content of these leaves which is responsible for such an effect. On the contrary, in vitro studies carried out by Bhade (1984) and Chawla *et al* (1988) in our laboratory have demonstrated a 15% reduction in iron availability on addition of fenugreek leaves, which are very low in oxalate (only 0.014%), to cereal meals. These findings support the assumption that some factors, other than oxalate, that are present endogenously in green leafy vegetables, may be responsible for making the iron less available for absorption (Gillooly et al, 1983). Examples of such factors are polyphenolic compounds, fibre and calcium salts, that may exert an inhibitory efect on iron availability by forming insoluble complexes with iron, in combination with oxalates, thereby reducing its availability from food.

Mechanism of action : The mechanism by which oxalate may influence iron absorption is not clear. However it is speculated that under certain conditions oxalate may act as a ligand and form insoluble chelates with iron, resulting in a decreased availability of iron (Hazell, 1985). Like phytate, the effect of oxalate on iron availability may also be related to the type and proportion of other ligands present concomitantly in the meal.

In view of the ambigous role of oxalate in modifying bioavailability or iron, it seems that the type of iron-oxalate chelate formed and its interaction with other lignads present in the medium may have a greater influence on iron availability than the presence of oxalate per se.

Calcium and Phosphate

Among inhibitors of iron, calcium and phosphate salts have been studied quite extensively. Earlier studies on calcium and phosphate were carried out using animal models and they indicated that when added in large quantities, calcium or phosphate compounds decreased the availability of nonheme iron in rats (Hegsted et al, 1949; Greig, 1952; Chapman and Campbell, 1957). Subsequent studies on effect of calcium and/or phosphate on iron absorption have revealed mixed results, as indicated in Table 7. Since there are no in vitro studies for comparison with the in vivo studies, this section deals with the effect of calcium or phosphate per se, as against their combined effect. This is

TABLE 7

Effect of calcium/phosphate on iron absorption in the in vivo human/rat studies

	Reference	Type of study	Form/Quantity of Ca/P	Effect on iron absorption	Direction and Quantum of effect
1.	Buttner and Muhler, 1959	In vivo (Rat model)	Fe - 50 mg (as $FeSO_4$)	Liver iron content 210 mg%	-
			P - 50 mg (as NaH_2PO_4) + Fe - 50 mg (as $FeSO_4$)	120 mg%	43%↓(by P)
			P - 100 mg + Fe - 50 mg	100 mg%	52%↓(by P)
2.	Peters at al, 1971	In vivo (Human study)	Sodium-postassium phosphate salts	Almost nil absorption	Nearly 100%↓ (by P)
			Phosphate salts with egg white	-	absorption↓ by one-third (by P)
			egg yolk or vitallin (phosphoprotein)	Almost nil absorption	Nearly 100%↓ (by P)
3.	Cook at al, 1973	In vivo (Human study)	Ferrous sulfate	taken as 100%	-
			Na-iron pyrophosphate	10%	One-tenth of the ferrous sulfate value (↓by P)
		In vivo (Human study	Ferric-ortho-phosphate	33%	One-third of FeSO4 value for iron absorption (↓by P)

Table 7 contd..

4.	Monsan and Cook	In vitro (Human study)	Basal meal (semi-synthetic) SS	2.2%	-
			SS + Ca (202 mg) as $CaCl_2$	1.5%	32% ↓ (by Ca)
			SS + P (404 mg) as K_2HPO_4	1.5%	32% ↓ (by P)
			SS + CaP (202 Ca + 414 P)	0.6%	73% ↓ (by Ca & P)
5.	Snedeker et al, 1982	In vivo (Human study)	Moderate Ca (as Ca - gluconate; 780 mg/day) + Moderate P (as glycerol phosphate; 843 mg/day)	Apparant Absorption 4.1%	No significant reduction
			Moderate Ca+ High P (2442 mg/day)	5.7%	No significant reduction
			High Ca High P (2382 mgCa & 2442 mgP per day	1.2%	iron retention ↓ but statistically non-significant (No effect by CaP)
6.	Barton et al, 1983	In vivo (Rat model)	2 mM $FeCl_3$	33.6%	-
			2 mM $FeCl_3$ + 200 mM $CaCl_2$	10.4%	70% ↓ (by Ca)

Table 7 contd...

7.	Dawson-Hughes et al, 1986	In vivo (Human study)	Standard meal (3.6 mg Fe)	6.3%	-
			Meal + Ca (500 mg) as $CaCO_3$	43% of the control	57%↓(by Ca)
			Meal + Ca (500 mg) as Hydroxy-apatite	46% of the control	54%↓(by Ca)
8.	Mehansho et al, 1987	In vivo (Rat model)	Calcium	reduction in the amount of iron transferred to systemic circulation	↓by Ca
9.	Dheer et al, 1990	In vivo (Human study)	Placebo (238 mg Ca)	8.3%	
			Placebo + milk (446 mg Ca)	3.4%	80%↓(by Ca)
			Placebo + Ca-citrate-malate salt (450-550 mg Ca)	6.8%	26%↓(by Ca)
			Placebo + orange & CCM juice (541 mg Ca)	7.4%	Non-significant
10.	Cook et al, 1991	In vivo (Human study)	Enhancing meal (Hamburger & bun) (5.1 mg Fe)	13.4%	-
			Enhancing meal + Calcium salts (600 mg)	8.2% to 11.9% (by Ca)	11% to 39%↓
			Inhibiting meal (egg, muffin, bran flakes, milk & coffee) (4.7 mg Fe)	1.2%	
			Inhibiting meal + Calcium salts (600 mg)	0.4% to 0.7%	42% to 63%↓ (by Ca)

Table 7 contd . . .

11. Hallberg et al, 1991	In vivo (Human study)	Wheat bun (3.8 mg) Fe	18% to 22%	-
		+ 40 mg Ca	12.8%	40% ↓
		+ 75 mg Ca	11.2%	45% ↓
		+ 165 mg Ca	8.8%	60% ↓
		+ 300 mg Ca	6.4%	70% ↓
		+ 600 mg Ca	3.6%	80% ↓
		Wheat bun + milk (165 mg Ca)	7.2%	57% ↓
		Wheat bun + milk (165 mg Ca)	13.6%	45% ↓
		Hamburg meal + wheat bun (containing heme iron)	15.6%	-
		Hamburger meal + wheat bun Ca (165 mg)	11.6%	26% ↓

followed by dose effect of calcium and the mechanism of action of these two factors.

Effect of calcium and/or phosphate :- What emerges from the studies, described in Table 7, is that the inhibitory effect of calcium or phosphate shows considerable variation. While some studies have demonstrated an antagonistic effect of large quantities of phosphate on iron absorption (Buttner and Muhler, 1959; Peters et al, 1971; Cook et al, 1973), others have found no significant inhibitory effect of phosphate per se (Monsen and Cook, 1976; Snedeker et al, 1982). Similarly, with respect to calcium also, there are some studies that show reduction in iron absorption on addition of calcium (Barton et al, 1983; Dawson - Hughes et al, 1986;,Dheer et al, 1990) while others indicate that presence of calcium per se in the meal does not alter iron absorption significantly (Monsen and Cook, 1976, Snedeker et al, 1982). However in a recent study by Cook et al (1991), it was demonstrated that all types of calcium supplements (calcium carbonate, calcium citrate and calcium phosphate) strongly inhibited nonheme iron absorption, when consumed with a high bioavailability or a low bioavailability meal by 61 human volunteers. The inhibition was less marked from the high iron availability meal (28%) than from the breakfast meal of low iron availability (55%). Moreover, in all their experiments, calcium phosphate was found to be most inhibitory in nature while calcium carbonate was least inhibitory if taken with water. Based on their findings, the authors suggested that if calcium supplements have to be taken along with iron (for better

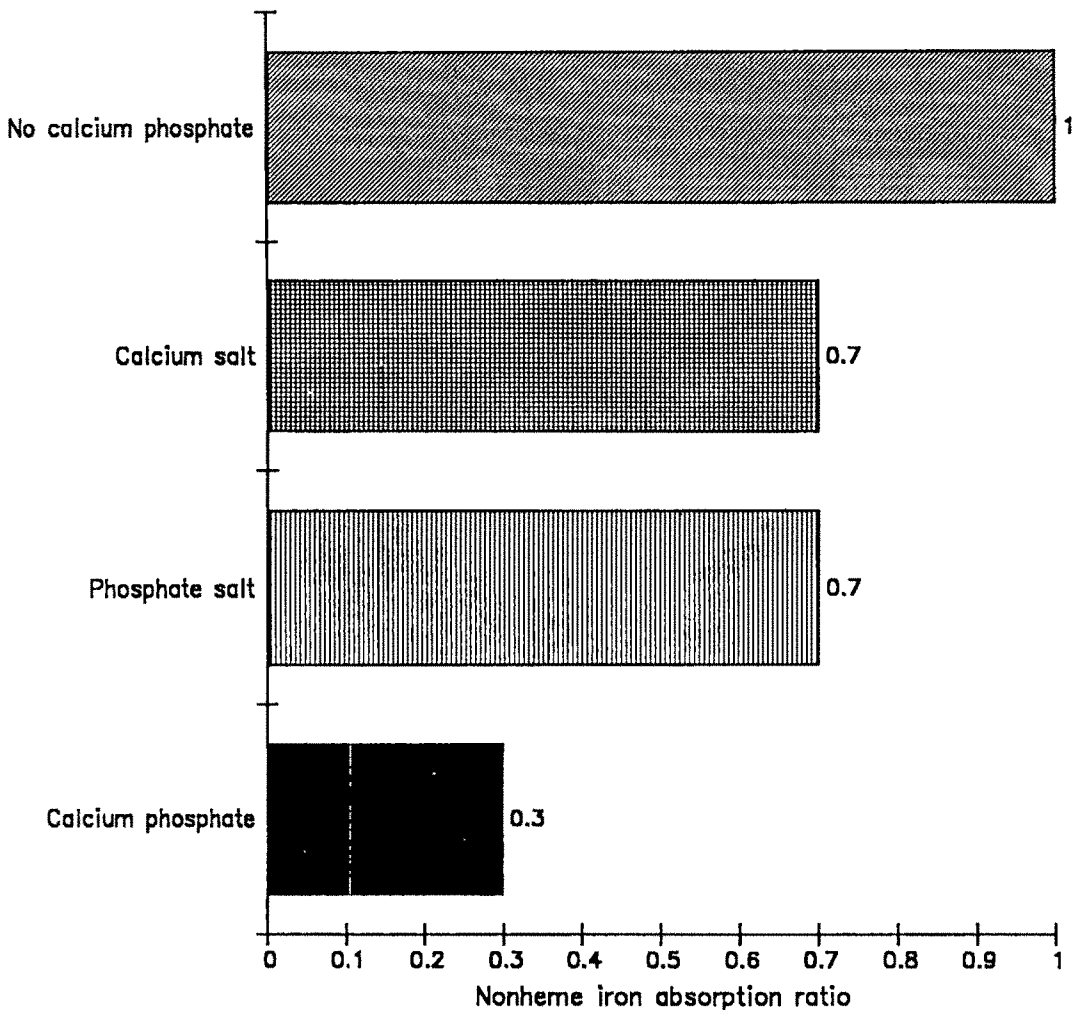
compliance), it would be better to use calcium carbonate, and that the supplements should be taken between meals rather than with the meals. Earlier studies by Monsen and Cook (1976) (Figure 9) and by Peters et al (1971) support the findings of the more recent study by Cook et al (1991) of the stronger inhibitory effect of calcium and phosphate when present together as against either of them present alone.

Though Snedeker et al, (1982) have also indicated that high calcium along with high phosphate in the diet may have a more inhibitory effect on iron absorption than high phosphate fed alone with the diet, they could not demonstrate any statistical significance for their observations. Towards an explanation for this, the authors suggested that since the calcium and phosphate used in their study were organic compounds (Ca-gluconate and glycerol phosphate respectively) and not inorganic salts they might not be as immediately available for forming a complex with iron in the intestinal lumen, as the inorganic salts. Hence, some amount of iron might still be absorbed inspite of the presence of these two inhibitory compounds in the diet.

Dose effect of Calcium : Hallberg et al (1991) recently reported an inhibitory effect of increasing doses of calcium on nonheme as well as heme iron absorption in 126 human subjects. The authors used calcium chloride as the calcium supplement and found that increasing doses of 40 mg upto 600 mg calcium resulted in a progressive fall in nonheme iron absorption (from 22% to 3.6%). Further, addition of 165 mg calcium in the form of

Figure 9

Decreased absorption of nonheme iron in healthy adult men who were presented with 700–kcal semi–synthetic meals with the addition of calcium and/or phosphate salts. Absorption of nonheme iron was set at 1.0 in meals to which no calcium or phosphate was added (Reproduced from Monsen, 1988; Data from Monsen and Cook, 1976).

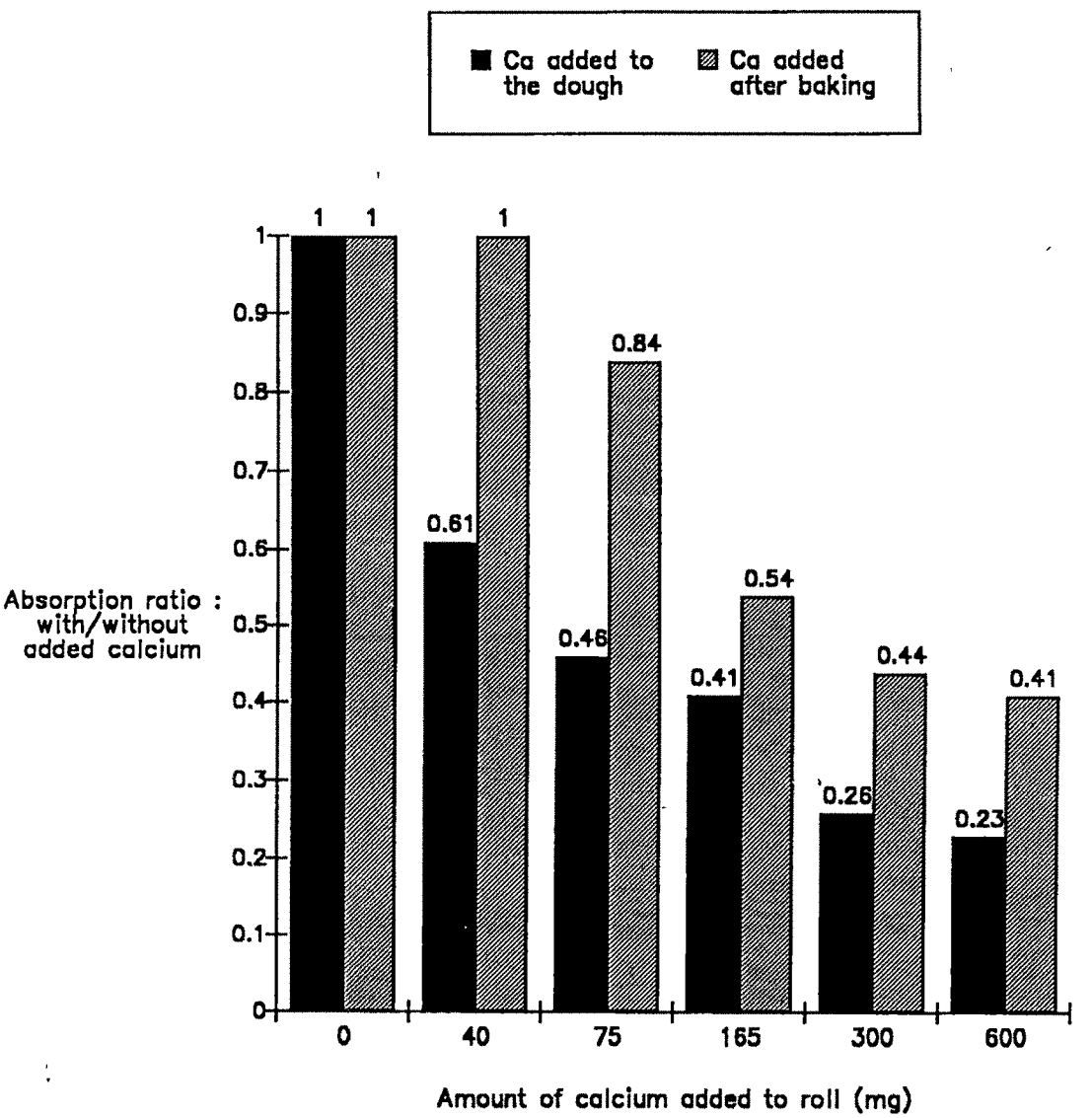


milk or cheese also brought about similar inhibition (46 to 57%) as that observed with the same quantity of $CaCl_2$ (60%). The authors also reported a marked reduction in heme iron absorption on addition of 165 mg $CaCl_2$, when added to a hamburger meal. An interesting and nutritionally significant finding in the above study was that addition of calcium to the dough before baking the rolls was much more inhibitory than when it was added to the rolls after baking. The authors, by estimating phytin-phosphorus content of the two sets of rolls, found that addition of calcium prior to baking decreased phytate degradation (which normally occurs during baking). They have suggested that the higher amount of phytate in these rolls contributed to an increased inhibition of iron absorption. In contrast, when calcium was added to the rolls after baking, there was a significantly lower concentration of resultant phytate in the rolls, as also a lower inhibitory effect on iron absorption, as shown in Figure 10. These results imply that the inhibition observed in this study is a combined effect of increased amount of phytate in the meal as well as a direct effect of calcium on iron absorption.

Mechanism of action : The exact mechanism by which calcium and phosphate impair iron absorption is not very clear. Initially, it was suggested that in the presence of phosphate, iron is converted to ferric-phosphate which is precipitated and thereby made unavailable for absorption (Buttner and Muhler, 1959; Peters et al, 1971). In the presence of both calcium and phosphate, a more effective reaction results in the formation of

Figure 10

Effect of calcium ($CaCl_2$) on iron absorption from wheat rolls, expressed as the ratio of with to without added calcium to the rolls. Calcium was added either to the baked rolls or to the dough. (Reproduced from Hallberg et al, 1991)



$Ca\text{-}H\text{-}PO_4$ which in turn combines with iron and removes it from the solution.

However, Barton et al (1983) later suggested on the basis of isolated gut loop experiments in rats, that calcium seems to reduce absorption of iron by competing, partially, for iron receptor sites at the intestinal mucosal level, thereby diminishing the mucosal uptake of iron. Secondly calcium may also influence the transfer of iron into the systemic circulation adversely. The authors further suggested that the inhibitory effect of calcium depends not on the molar ratio of calcium to iron, but upon the absolute quantity of calcium present in the intestinal lumen.

Recently, Mehansho et al (1987) reported, on the basis of a number of short and long term, isolated duodenal loop experiments in rats, that it is the transfer of iron to the serosal side, which is interrupted by presence of calcium and not the mucosal uptake of iron by the cells. Hallberg et al (1991) reinforced this hypothesis by indicating that calcium inhibited both nonheme as well as heme iron absorption in humans. Since these forms of iron share a common pathway only in the final transport step, from the mucosal cell to plasma, the authors suggested that there could be competitive inhibition by calcium on intracellular transfer of iron. They further indicated that since the inhibitory effect of calcium was dose-related upto a maximum inhibition of 60% observed with 300 to 600 mg Ca, it is possible that calcium did not completely block iron absorption but influenced it by interaction in the transfer step of the mineral.

Other factors

There are many other factors which may influence iron absorption but the role of these has not been well documented yet. These include certain organic acids such as malic acid and tartaric acid, which are reported to influence iron absorption favourably (Gillooly et al, 1983), fibre (Monnier et al, 1980; Faraji et al, 1981), soy products (Cook et al, 1981; Schricker et al, 1982; Lynch et al, 1982; Hallberg and Rossander, 1984; Macfarlane et al, 1990), egg yolk (phosvitin) and egg albumen (Peters et al, 1971; Cook and Monsen, 1976; Monsen and Cook, 1979) and EDTA, (Cook and Monsen, 1976), all of which have been shown to reduce iron absorption.

Prediction of Iron Availablility - Recent Advances

Accumulating evidence has suggested that a number of enhancers and inhibitors interact to determine the net bioavailability of iron from a given meal (Monsen, 1988). Therefore, in order to calculate the amount of iron absorbed from different meals, one must consider the nature and proportion of all these factors ingested concomitantly, to arrive at a relatively accurate estimate of iron adequacy of the meals.

Attempts have been made in the literature to estimate available dietary iron on the basis of the amount of heme iron and its availability as well as the amount of nonheme iron and its availability, (Monsen et al, 1978). In a subsequent study, this model has been refined further, through calculation and

computerization, to arrive at a prediction equation for estimating the per cent bioavailable dietary iron for an individual with 500 mg body iron stores (Monsen and Balintfy, 1982). This model involves calculation of the total iron content, amount of heme iron, amount of nonheme iron and bioavailable nonheme iron as influenced by the total content of enhancing factors (ascorbic acid, meat, fish or poultary) present in a meal or snack. Summing up these fractions of available iron from each meal of the day, a quantitative estimate of the total bioavailable iron in a day can be arrived at. The adequacy of the day's diet can therefore be assessed by comparing this value with the recommended daily allowance for absorption of iron in a day.

The model of Monsen and Balintfy (1982) has been utilized in a recent survey, to estimate the bioavailability of iron from meals and snacks consumed daily by 224 adolescents using 24 hr food records (Viglietti and Skinner, 1987). Calculations revealed that the mean available iron from boys' diets (1.38 mg) and from girls' diets (0.91 mg) was below the recommended levels (1.6 mg and 1.4 mg respectively), calculated on the basis of 10% net availability of typical American meals (Food and Nutrition Board, 1980). The authors also observed that the diets that met RDA for available iron had unusually high quantities of energy (1.5 times RDA), ascorbic acid (2 times RAD) and animal tissue (3 times RAD). In cereal based vegetarian Indian meals where the inhibitors are a common occurrence and which do not contain

appreciable amounts of enhancers, application of the above model becomes limited.

There is a need, therefore, to evolve a model, based on the interaction effect of various enhancers and inhibitors, in order to assess the adequacy of vegetarian diets with respect to iron availability. Further, to increase the quality of the existing diets in terms of bioavailable iron, it becomes imperative to have a quantitative estimate of the levels of enhancers required to achieve a desired level of bioavailable iron from a diet containing known amounts of inhibitors.

Addressing the above needs, it was planned in the present study, to integrate the interaction effect of various enhancers and inhibitors into a practical model for predicitng iron availability from cereal based typical Indian meals.