THE EFFECT OF CALCIUM ON THE ABSORPTION OF IRON BY HUMAN BEINGS

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By

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PREFACE

With the modern trend toward the enrichment of foods with minerals and vitamins and the frequent use of capsules to supply these food essentials, the question of what effect an excess of one mineral may have on the metabolism of another has been brought to the attention of those interested in nutrition research.

As iron and calcium both play an important part in human nutrition and are two of the elements most frequently given as the pure salts, the effect of large amounts of calcium salts on the absorption of iron in human beings was chosen as the problem for this study. The number of subjects used is too limited to permit definite conclusions, but the methods established and the results presented may lay the foundation for more extensive work along this line.

A review of the experimental work which has been done on the role of iron in animal nutrition and its importance in the blood picture of human beings is presented as a preliminary study to the experimental portion of this paper.

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INTRODUCTION

The absorption and metabolism of iron and its function in the body have for many years been subjects of great interest to the biochemist and the nutritionist. The importance of the role played by iron in the animal body was expressed by Sherman (63) in 1907 in his statement that iron is

> an essential constituent of the cells and tissues most directly concerned with the processes of oxidation, secretion, reproduction and development and stands therefore in fundamental relation to metabolism and nutrition in the broadest sense.

There are many reports in the literature of studies which have been made on the various phases of iron metabolism. As will be noted in the literature review which follows, the major portion of these studies have been carried out with small laboratory animals as experimental subjects. These studies have included investigations of hemoglobin regeneration, the biological availability of various forms of iron and the relation of other substances such as copper to hemoglobin formation. The extent to which information obtained with small animals can be applied to human beings is not known. Some investigations have been carried out with human beings in an attempt to establish the iron requirements and normal hemoglobin values for man. There have also been several studies made on the treatment of pathological conditions connected with iron metabolism.

In 1939 Ammundsen (1) reported some studies which indicated that human blood contains forms of hemoglobin in addition to the molecule which so readily combines with oxygen. Attention has also been directed to the forms of non-hemoglobinous iron present in the blood which include the plasma or serum iron and the "easily split-off" iron thought

to be present in the red blood cells. According to Barkan (10), 1927, these two forms of iron make up the transport iron system in the body.

Because of the small amount of non-hemoglobinous iron in blood serum, its physiological importance had not been studied until Moore, at the University of Ohio, and Tompsett, of the Royal Infirmary at Glasgow, recently carried out a number of studies on this iron fraction. In 1937 Moore, Doan and Arrowsmith (49) showed that the oral administration of large amounts of iron salts produced a rise in the serum iron of human beings within a few hours. The large amount of iron which this worker found necessary to administer in order to produce any observable rise in serum iron limits the adaptability of the method for measuring the availability of iron in foods. However, the use of the measure of serum iron to determine the effect of other salts on the absorption of iron seems feasible.

Kletzien (37), in 1940, demonstrated that the amount of iron absorbed by anemic rats on a diet containing 0.5 mg. of iron and 0.02 mg. of copper was reduced by the addition to the diet of 1 per cent or 3 per cent calcium carbonate or calcium lactate. At the end of a five or six weeks' feeding period the rats given the calcium supplements showed a lower iron content in all tissues of the body except the spleen than did the anemic control animals. The depressive effect of the calcium lactate was more marked than that of the calcium carbonate. Other calcium salts, with the exception of calcium sulfate, produced similar effects.

Anderson, McDonough and Elvehjem (4) reported, in 1940, a maximum hemoglobin regeneration and iron storage in rats on a diet containing a calcium-phosphorus ratio of 0.45. As the calcium-phosphorus ratio increased above this value the amount of iron stored in the body decreased.

In 1939 Crowley and Taylor (16) found that a group of non-rachitic, anemic children which failed to respond to iron therapy had a normal blood serum calcium level but a low phosphorus level. Upon the administration of large amounts of vitamin D the serum phosphorus level was raised to normal and the anemia then responded to the iron therapy. The authors concluded that sufficient vitamin D appeared to be necessary for the proper utilization of iron.

In view of these observations which have been made on the effect of calcium, phosphorus and vitamin D on the metabolism of iron, the present study was planned to determine the effect of a high calcium intake on the absorption of iron by human subjects. As it has been shown previously by Moore and coworkers (49,51) that large doses of iron salts produce a marked rise in blood serum iron, it seemed probable that if calcium has a depressive effect on iron absorption this rise would not occur following the simultaneous oral administration of iron and calcium salts.

REVIEW OF LITERATURE

It is evident from the following review of the literature that although certain facts relative to iron metabolism are generally accepted, there is still much controversy concerning some phases of the subject.

According to Sherman (63), 1907, the available iron in ingested food is absorbed mainly from the duodenum and, to some extent, from the jejunum. It enters the blood stream and is carried as transport iron in the blood plasma to the storage depots--the liver, the spleen and the bone marrow (48). When needed for the formation of hemoglobin in the red blood cells, the iron is drawn from these storage depots and is carried again as transport iron to the bone marrow where red cell formation takes place (32, 48, 62).

Schultze (62), 1940, believes the hemoglobin is formed in the nucleus of the developing red blood cell. The reticulocyte then either resorbs or ejects the nucleus before entering the circulating blood. The length of life of the mature erythrocyte in the blood stream is not known. In 1937 Isaacs (32) reported estimates varying from 4 to 120 days.

Following the breakdown of the erythrocytes, the greater part of the iron is used again by the body for new cells, but a small fraction of it is eliminated each day. A fixed amount of about 0.2 mg. per day passes from the body in the urine, and amounts varying from 5.5 to 12.5 mg. per day are excreted through the walls of the lower intestine and eliminated with the feces (63).

According to Schultze (62) Hahn and Whipple (25), Josephs (34) and Schultze, Elvehjem and Hart (61) have reported that different iron compounds in the tissues of the body show variations in the ease with which they are mobilized to become available for hemoglobin regeneration. In

even the most severe cases of anemia not all of the iron of the liver and the spleen becomes available. None of the iron of the heart is ever mobilized for hemoglobin regeneration.

Leverton (42) recently, 1941, found that over extended periods of low iron intake by human subjects, the level of the serum iron dropped below normal and then rose immediately when adequate iron was added to the diet. This observation might indicate that the serum iron is the first to be mobilized for hemoglobin regeneration when the dietary intake is inadequate.

The most satisfactory results in the studies of iron metabolism have been obtained when rats or rabbits made anemic on a milk diet served as experimental animals. Abderhalden (2, 3) demonstrated in 1900 that rats developed anemia when kept on a milk diet, and that the addition of inorganic iron salts alone did not alleviate the condition. He believed this to be evidence that the body was unable to form the porphyrin ring and that it, therefore, must be present in the diet. These results but not the conclusions were substantiated by some early experiments of Hart, Steenbock, Elvehjem and Waddell (28, 29),in 1925 and 1927, in which they observed that rabbits made anemic on whole milk did not regenerate hemoglobin when ferric oxide alone was added to the diet. However, upon the daily addition of five grams of ground, dried lettuce leaf or larger amounts of dried, ground cabbage to the milk and ferric oxide diet, these authors were able to produce complete hemoglobin regeneration in the anemic rats within thirty days. In further investigations in 1928, these same workers (30) found that it was the copper present in the vegetable and liver ash used as a supplement to the milk and iron salt diet which produced the hemoglobin regeneration. They proposed the theory that copper acts

as a catalyst for iron in the formation of hemoglobin.

To study the comparative availability of organic and inorganic iron for the treatment of nutritional anemia, Elvehjem (18), 1932, divided rats made anemic by a milk diet into groups and gave each group either ferric chloride, purified hematin, ferric chloride plus copper, or hematin plus copper. In the absence of copper the hematin iron was as ineffective as the ferric chloride for the treatment of anemia. The hemoglobin content remained at from six to seven grams per 100 cc. of blood as long as hematin and copper alone were fed, but when ferric chloride and copper were given the hemoglobin rose to 17 grams per 100 cc. of blood. The iron content of the livers of the rats was not increased by hematin iron in the diet, which indicated to the authors that the decreased activity of the organic iron was due to the inability of the intestinal tract to absorb it. In cases of pernicious anemia, inorganic iron and copper alone were not sufficient to relieve the condition. Some unknown substance present in liver was also necessary.

Rose and Vahlteich (55) found in 1932, that 0.2 mg. of iron furnished by whole wheat, bran or oat meal produced complete hemoglobin regeneration in anemic albino rats within six weeks. White flour with copper and iron added to it to make it equal to whole wheat in respect to these two minerals did not give as good regeneration as did the whole wheat. Further studies by Rose and Kung (56) during the same year showed that dried liver gave even more rapid hemoglobin regeneration than did whole wheat.

The discovery of the role of copper in hemoglobin formation invalidated much of the work which had previously been done on the availability of iron in foodstuffs as determined by biological methods since the diets used prior to the discovery may not have supplied sufficient copper.

The a-a' dipyridyl method introduced by Hill (31), in 1930, for determining non-hematin iron has been found useful by some workers (19, 65, 67) in differentiating between available and non-available iron in foods The dipyridyl reagent will not react with iron that is bound in the complex hematin molecule. It is this same iron which is not available to the animal body for hemoglobin regeneration due to the inability of the intestinal tract to breakdown the porphyrin ring so the iron can be absorbed (18).

The term "ionogenic iron" was suggested by Free and Bing (23), 1940, in preference to "available", "inorganic" or "ionizable" iron. They define their term as the iron in foods which reacts when treated with the dipyridyl reagent of Hill (31) or, according to Shohl's (66) terminology, "yields ions in the course of body processes." In the studies made by Free and Bing on wheat of various varieties it was found that an average of 81 per cent of the total iron in wheat was ionogenic iron according to the dipyridyl method. By biological assay, the iron of wheat proved to be fully as available to the rat for hemoglobin regeneration as an equal amount of iron in the form of ferric chloride.

In 1935 Elvehjem, Hart and Sherman (19) studied the availability of iron in wheat, oats, yeast, ferric chloride, ferric pyrophosphate, iron hypophosphite, iron glutamate and glutamic acid parahematin by the dipyridyl method and also by biological assay. They found the iron in the wheat, oats and yeast to be about 50 per cent available by both methods. All of the salts except glutamic acid parahematin were approximately 100 per cent available as determined by both chemical and biological methods. The iron in the glutamic acid parahematin showed very slight availability by animal feeding assay methods and failed to react with dipyridyl reagent.

Further work in 1934 by these same men (64) showed a like correlation between the results obtained by biological and chemical assay methods when applied to beef and pork liver, beef and pork muscle, soy beans, oysters, spinach, alfalfa and blood.

The work of Ascham, Speirs and Maddox (5,6), in 1938 and 1939, did not substantiate that of Elvehjem, et al, on the relation between the ionizable or non-hematin iron and that available for hemoglobin regeneration. Because of the color of the solution from the plants studied, Ascham found it necessary to modify the dipyridyl method used by Elvehjem. This may account, in part at least, for the differences in the results obtained by the two groups. The experiments of Ascham and co-workers showed that the iron present in blackeyed peas, green and yellow split peas, navy beans, pinto beans and butter beans was as available for hemoglobin regeneration in anemic rats as the same amount of iron in the form of ferric chloride when each was tested by the biological assay method. In all of their studies these authors supplied 0.3 mg. of iron per day for six weeks as had been recommended by Elvehjem and co-workers (19). However, in 1937 Smith and Otis (67) found 0.3 mg. of iron per day to be in excess of the minimal requirements for complete hemoglobin regeneration in six weeks. They obtained normal hemoglobin levels in anemic male rats with 0.25 mg. of iron per day in the diet and in female rats with 0.20 mg. of iron per day. There was close agreement between biologically available and ionizable iron in most of the foods they studied. If the amount of iron fed by Ascham, et al, was in excess of the minimal requirements of their animals, a value for available iron would have been obtained that was higher than the actual food iron available for biological use.

In 1939 McCance (44) pointed out, in a review of the work done on

ionizable and available iron in foods, that the wide discrepencies in the methods used by different workers makes comparisons difficult. He further recommended that much more work needs to be done on the relation of food iron to man.

Schultze, Elvehjem and Hart (59, 60), 1934, studied the availability of copper in foods and in copper salts for utilization in hemoglobin formation. The copper contents of copper caseinate, glycine amide biuret, alanine amide biuret and hemocyanin from limulus polyphemus were all utilized. Copper from hematoporphyrin was not utilized even when fed at high levels. Wheat germ, alfalfa, brewer's yeast, pork heart and pork liver were also tested for the availability of their copper content. In all cases the copper was found to be readily utilized. The Wisconsin workers concluded that unavailability of dietary copper was of little practical importance since copper is widely distributed in a utilizable form in foods common to the human diet.

Rose, Vahlteich and MacLeod (57) found in 1934 that although eggs were very high in iron they did not give good hemoglobin regeneration when used as the only source of iron in the diet of anemic rats. They believed the limiting factors in the egg yolk to be the amount of copper present, the form of the iron and some other factor which they were unable to identify. This third factor in the egg yolk which limited it as a source of iron for hemoglobin regeneration was soon shown by Sherman, Elvehjem and Hart (64), 1934, to be the sulfur which is present in large quantities in egg yolk. The copper combines with the sulfur forming copper sulfide in which form it is not absorbed by the intestinal tract and therefore the copper is not available to catalyze hemoglobin formation.

In order to determine the factors influencing the absorption of iron

and copper from the alimentary tract, Tompsett (73), 1940, placed 5 cc. of the mixture to be tested plus 1 mg. of iron in the form of a salt in a parchment thimble and dialyzed it against 15 cc. of water for four hours. The solutions tested were: 1, egg yolk and egg white suspensions; 2, peptic digests of the egg yolk and egg white; 3, neutralized solutions of the peptic digests. In the first case ferrous iron dialyzed readily from both egg white and egg yolk, but ferric iron was not dialyzed from either. In the second case ferrous iron dialyzed readily from both, and ferric iron dialyzed from the egg white. In the third instance both forms of iron dialyzed from the egg white, but there was no dialysis from the egg yolk. The same experiments carried out in vivo with rats gave similar results. The author concluded from these findings that the absorption of iron from the intestinal tract can take place only when the iron is in the ferrous state. Reducing substances in the diet will reduce the iron to the ferrous form in the presence of gastric acidity. If the diet is higher in oxidizing agents than in reducing agents, a large amount of the iron will be in the ferric state and therefore cannot be absorbed. Tompsett considers that it is impossible to state any particular quantity of dietary iron as being sufficient for animal needs since the degree of iron absorption is dependent on so many factors.

It can easily be seen from the work thus far reviewed that most of the iron metabolism studies have been carried out by measuring hemoglobin regeneration in anemic animals. This method naturally limits the controlled experimental studies to small laboratory animals with the exception of a few clinical cases of human anemia. However, hemoglobin determinations on human beings are used as a method of measuring nutritional condition with respect to iron and in the diagnosis of certain diseases.

Meyers and Eddy (46), 1939, pointed out that as each of the several methods now in use for the determination of hemoglobin values has a different level of blood hemoglobin content considered as 100 per cent, much confusion has arisen as to what constitutes a normal hemoglobin level. The present tendency is toward the use of the Newcomer (52) acid-hematin method by which the grams of hemoglobin per 100 cc. of blood can be determined directly. The calibration for this method is based on the oxygen capacity of the hemoglobin in blood.

Dowden and McNeil (17), in 1934, recommended the reporting of hemoglobin in grams per 100 cc. of blood instead of the many methods based on percentage values. They also believed that in view of the fact that there was a wide variation found in the hemoglobin values for healthy human beings, normals should be given as a range rather than a set average figure.

In a recent review, 1939, of work done on the hemoglobin content of human blood, Myers and Eddy (46) gave average figures of 15.8 gm. of hemoglobin per 100 cc. of blood for males and 12.8 gm. per 100 cc. for females as determined by the oxygen capacity method. These values were obtained by averaging the reports of a large number of workers in scattered localities.

There has been considerable controversey as to the cause of the normally lower hemoglobin values found in females than in males. Rose and Hubbell (58), in 1938, depleted the iron stores of both male and female rats to a uniform state of anemia then gave doses of iron and copper calculated each week to the individual animals weight. The iron was given until the hemoglobin reached a level of 14 gm. per 100 cc. of blood, and then the bodies of the animals were ashed and analyzed for

total iron. At the end of the depletion period the bodies of the control rats contained a uniform percentage of iron per gram of body weight regardless of sex, but at the conclusion of the iron feeding period the females had stored about 12 per cent more iron than the males when the intake was the same per gram of body weight. According to the authors these results showed that the female of the species had greater ability to store iron than the male.

In 1937 Leverton and Roberts (40), in a study of four healthy young college women, found that the actual loss of hemoglobin due to the menstrual period was not great, and varied from 14.26 to 22.84 mg. of iron per period in the 16 periods studied. These workers believed that the low hemoglobin values found in women may be due to habitually low intake of iron rather than loss during menstruation.

A number of attempts have been made to correlate the hemoglobin content with the total iron of the blood. Josephs (35), 1934, found the total iron to be consistently higher than the iron accounted for by the hemoglobin. The difference was not more than 10 per cent in normal subjects but was somewhat higher in certain pathological conditions.

Klumpp (38) likewise reported, in 1935, that attempts to correlate values of the hemoglobin content and oxygen capacity of the blood with determinations of total iron were of little value as the non-hemoglobin iron was a significant fraction of the total iron.

Myers and Eddy (46), however, in their work obtained values for the hemoglobin content of the blood by the acid hematin method, total iron determination and the benzedine method which agreed so closely that they concluded it legitimate for clinical use to calculate hemoglobin content from figures for total blood iron.

Johnson and Hanke (33), 1936, found the oxygen capacity and the total iron content of dog blood to agree within one per cent. They determined the iron by an iodometric method.

Three reasons are given by Farrar and Goldhamer (20) for the lack of information from controlled experiments on the iron requirements of normal adults. These are as follows:

> the difficulties encountered in the analysis of iron in biological materials, the small amount of iron metabolized by the human organism and the impossibility of obtaining a palatable iron-free or even a very low iron containing diet which is otherwise adequate for prolonged human consumption.

In addition to these factors, much of the early work done on iron requirements did not consider the fact that all iron in foodstuffs is not available to the human organism for hemoglobin regeneration.

In 1935 Farrar and Goldhamer (20) studied four healthy young adults on well controlled diets low in iron. One of the male subjects was on an iron intake of 4.9 mg. per day for 316 days. During the last 31 days he was in iron balance on a diet containing 5.2 mg. of iron per day. Two other male subjects had normal blood pictures and were in iron balance after four and five months on diets containing 7.1 and 7.8 mg. of iron per day, respectively. A young woman on a diet containing 9.1 mg. of iron daily for over a month was in iron balance during the intermenstrual phase. From these observations it appeared that the daily requirements of the normal adult males did not exceed 5 mg. of available iron.

In 1940 Barer and Fowler (7) reported the following recommendations for daily intake of available iron which have been given by various investigators:

Lintzel	0.9 mg. per day (if no special demands)
Stockman and Greig	3.5 to 5.5 mg. per day
Sherman	3.5 to 5.5 mg. per day
Farrar and Goldhamer	5.9 mg. per day (male)
Vahlteich, et al.	6.1 to 5.2 mg. per day (women during intermenstrual period)

In a study of 42 patients, Fowler and Barer (21), 1939, found in cases in which there was a negative balance when the diet contained only 3.81 to 6.76 mg. of iron per day, that increasing the iron intake to 12.22 to 15.33 mg. per day produced a positive balance. They found that achlorhydria and hypochlorhydria interfered with the retention of dietary iron and necessitated a larger intake to maintain equilibrium.

Widdowson and McCance (75) found, in 1937, that increasing the iron intake of normal subjects in iron balance on a low intake did not increase the iron excretion. The authors concluded from this that the intestine had no power to regulate the amount of iron in the body by varying the amount excreted; therefore, the amount of iron in the body must have been regulated by the amount absorbed.

The most recent work on iron requirements is that of Leverton (42), 1941, in which she found in a study with four college girls that although a balance between intake and output of iron could be maintained on an intake as low as 3.5 mg. of available iron per day, the serum iron dropped below the normal level on this low intake. When the iron intake was increased to 6.5 mg. per day the serum iron again rose to a normal level. This author concluded that, to assure a good condition of iron metabolism, emphasis should be placed more on the completeness of the diet as a whole than on the intake of iron alone.

In recent years the use of radioactive iron for tracing the metabolism of iron in the body has proved useful. Hahn, Bale, Lawrence and Whipple (26) with the use of this isotope demonstrated, in 1938, that

anemic dogs absorbed much larger amounts of iron supplied in the diet than non-anemic dogs on the same diet. The isotope appeared rapidly in both the plasma and red cells of the anemic dogs. From this work the authors concluded that the absorption of iron is dependent on the nutritional need of the animal.

In 1940 Hahn, Bale, Ross, Hettig and Whipple (27) used radio active iron to study the exchange of iron from plasma to red cells. There was no dialysis of iron from plasma to cells or vice versa when oxalated blood was held at a temperature of 37°C. for 24 hours. This would indicate that the iron must go from the plasma to the bone marrow where the red cell formation takes place before it appears in the cells as hemoglobin.

Miller and Hahn (47), 1940, demonstrated that in vivo radioactive iron does appear in the circulating red blood cells following its feeding. It was found to appear in what is known as the "easily split-off" fraction of the iron of the cells. There was no obvious relationship between the amount of the radioactive iron in the cell and the time following the administration of the isotope. It may be that its rate of appearance depended on the need of the body for replacement of red cells.

The "easily split-off" iron fraction in which Miller and Hahn found the radio isotope has as yet not been clearly defined. It comprises from 5 to 10 per cent of the total blood iron and is easily split off in ionic form when whole blood is treated with dilute hydrochloric acid as described by Barkan (8) in 1927.

Barkan (12) also showed in 1933 that this fraction could be distinguished from the hemoglobin and inorganic iron of the blood by its failure to be absorbed on aluminum hydroxide. The greater part of the "easily

split-off" fraction was found in the red blood cells and only a very small amount in the plasma. However, the amount of "easily split-off" iron was independent of the hemoglobin iron. Practically all of the iron of the cell stromata was of the "easily split-off" fraction. Freshly prepared hematin crystals contained some of this detachable iron in a form which Barkan (9) believed to be an adsorbed impurity. Moore (48) suggested in 1937, that it may be the iron fraction of one of the rarer types of hemoglobin molecule which had recently been found to exist in human blood. Barkan's basis for believing it non-hemoglobinous in nature was its failure to liberate a porphyrin ring when split off. As yet, the structure of the molecule containing the "easily split-off" iron has not been proved.

The specific physiological function of "easily split-off" iron is also a controversial subject. Barkan (10), 1927, considered it a fraction of the transport iron which serves the dual purpose of carrying iron into the blood stream and of transmitting oxygen to the red blood cells. He believed that plasma iron served only to carry the iron from the point of absorption to the "easily split-off" fraction. Moore and coworkers (48, 49, 50), 1937 and 1939, disagreed with this theory for two reasons: 1, Plasma iron shows a decided increase following the oral administration of large doses of iron, whereas, the "easily split-off" iron shows no change unless the initial value is subnormally low. 2, In vitro there is no increase in the non-hemoglobin iron of plasma after incubation of whole blood for 24 hours at 37.5°C. Moore suggested that it may be possible for such an exchange to take place in vivo, but that it does not seem probable.

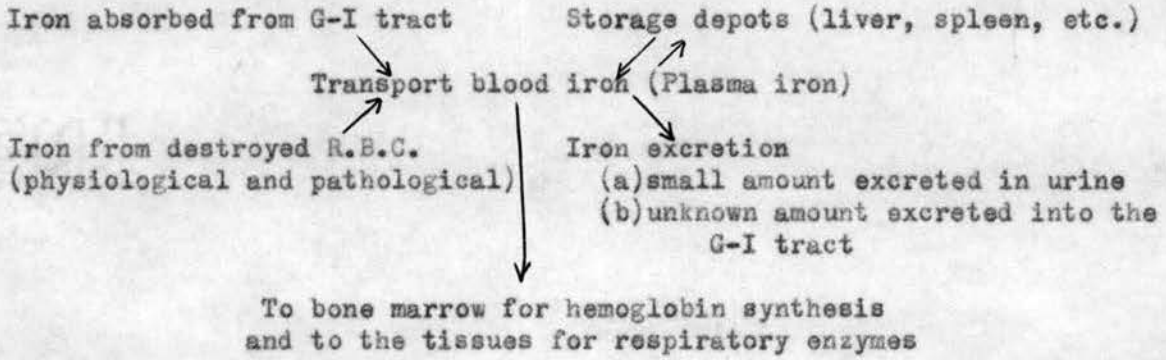
The third and last fraction of blood iron to be considered is the non-hemoglobin serum or plasma iron. Barkan (8) showed in 1927 that the

iron in serum or plasma was not ultrafiltrable, but it could be made so by acidifying with weak hydrochloric acid. After incubation for 24 hours at 37.5°C., the acidified plasma was submitted to a process of ultrafiltration. Under these conditions, the iron of the hemoglobin did not become ionizable, and the iron which passed through the filter was the non-hemoglobin plasma iron. In further experiments, in 1936, Barkan (13) found that human or horse blood rendered uncoagulable and allowed to stand for 24 hours did not show any dialysis of iron from corpuscles to plasma. Iron added to the plasma behaved as preformed acid-soluble plasma iron and could be recovered quantitatively from the plasma.

Moore, Doan and Arrowsmith (49), 1937, substantiated the work of Barkan and further showed that in vivo the amount of plasma iron present in peripheral blood was influenced by and was a measure of:

> (a) the amount of iron being absorbed from the gastro intestinal tract; (b) the extent and adequacy of the iron reserves of the body; (c) the ability of the bone marrow to utilize iron in the synthesis of hemoglobin; (d) the rate of hemoglobin synthesis; (e) the extent of hemolysis taking place in spleen and other tissues; and (f) the physiological or pathological equilibrium existing between (d) and (e).

The following chart from Moore (48) illustrates the interrelation between the transport iron and the other organs of iron metabolism.



Tompsett (72) further extended the study in 1940 by showing that ferrous iron added to plasma could be dialyzed out against water but ferric iron could not be. When the plasma was treated with hydrochloric acid according to Barkan's procedure, the ferric iron was reduced to the ferrous state and could then be dialyzed. Tompsett suggested that although the iron in the plasma of drawn blood was in the ferric state, that probably in vivo the iron was in the ferrous state at some place in the circulatory system. He believed that the plasma protein in the presence of acid was responsible for the reduction of the iron.

Inspection of the literature on plasma iron determinations reveals wide variation in the values observed. A summary of some of the results reported is given in Table I. With the exception of the first three listed, the workers obtained fairly close agreement in the plasma iron content. Moore (48) gives as a normal range for human subjects a plasma iron content of 0.050 to 0.180 mg. per cent. It will also be noticed on table I that the serum iron in normal males is approximately 0.020 to 0.030 mg. per cent higher than the mean value for normal females.

In summary the known iron constituents of the blood can be divided into three fractions: (a) hemoglobin iron, which comprises the greater part of the blood iron and is found in the red blood cells; (b) the "easily split-off" fraction, which is not well difined either as to structure or physiological significance but is believed to be present also in the red cells; and (c) the non-hemoglobinous plasma or serum iron, which composes at least part and perhaps all of the transport iron system. It is the latter fraction which is used in the experimental work of this study as a measure of the iron absorbed from the intestinal tract.

TABLE I**

Iron Content of Human Serum or Plasma

Investigators	Material Used	Subjects	No.	Results
1. Starkenstein and Weden (69)	Protein free serum (trichloracetic acid)	Adults	5	0.690*(0.570-0.790)
2. Riecker (53)	Protein free serum (trichloracetic acid)	Adults	40	1.110 (0.9-1.4)
3. Marlow and Taylor (45)	Protein free serum (defibrinated; trichloracetic acid)	Adults	5	0.400-0.700
4. Warburg and Krebs (74)	Whole serum. Catalytic method	Adults	4	0.084 (0.067-0.116)
5. Langer (39)	Protein free serum (trichloracetic acid)	Adults	20	0.110 (0.050-0.180)
6. Guthmann, Brückner, Ehrenstein and Wagner (24)	Whole serum (ultrafiltration)	Men	22	0.065 (0.042-0.098)
		Women	26	0.068 (0.035-0.098)
7. Locke, Main and Rosbash (43)	Protein free serum (trichloracetic acid)	Men	8	0.100 ±0.015
		Women	9	0.077 ±0.015
8. Barkan (11)	Whole plasma and serum	Adults	15	0.105* (0.056-0.140)
9. Fowweather (22)	Hemoglobin free whole plasma	Men	10	0.125 (0.095-0.180)
		Women	10	0.105 (0.060-0.156)
10. Tompsett (71)	Thiolactic acid-protein free serum	Adults	10	0.120-0.220
11. Thoenes and Aschaffenburg (70)	Protein free serum (trichloracetic acid)	Infants at birth	12	0.173 (0.134-0.291)
		Children 13 days to 9 yrs.	60	0.040-0.196
12. Roosen-Runge (54)	Whole serum--ultrafiltration	Adults	10	0.110
13. Moore (48)	Whole serum (Hemoglobin present quantitated)	Men	15	0.121 (0.094-0.174)
		Women	15	0.097 (0.058-0.142)

* Not normal subjects

** Moore (48)

METHOD OF PROCEDURE

This study was planned to determine whether or not large amounts of ingested calcium have any effect on the absorption of iron from the intestinal tract. Since Moore and coworkers (49) showed that the ingestion of large doses of iron salts produced a rise in the transport or blood serum iron, it was believed that the effect of the calcium could be determined by first measuring the serum iron following the administration of ferric ammonium citrate alone and then measuring it following the administration of ferric ammonium citrate and calcium lactate together. Calcium lactate was used as the calcium salt because of several salts tested it was shown by Kletzien (37) to have the greatest effect on depressing hemoglobin regeneration in rats. Ferric ammonium citrate was used as the source of iron because its high solubility renders it available for absorption (51).

The experiment was carried out on three healthy young women between the ages of 18 and 24 years. These subjects were attending college and participating in their normal activities as students. The days on which blood samples were taken, the subjects came to the laboratory before breakfast and stayed the entire period of about seven hours. After the first blood sample had been taken, the subject was allowed to eat as much white bread, butter, jelly and bananas as she wished throughout the day. These foods were selected because they had comparatively low calcium and iron contents and at the same time a high satiety value.

On the first day a blood sample was taken while the subject was in a fasting condition, and then the subject was given 5 gm. of ferric ammonium citrate orally in capsules. This quantity of salt supplied approximately 0.4 gm. of iron. Successive blood samples were taken

at 1, 2, 4 and 6 hours after the administration of the iron. The blood was analyzed for serum iron, total iron and hemoglobin. At least one week was allowed to elapse between the first experimental day and the second to assure the formation of new blood and to clear the blood system of the iron that had been taken on the first day. On the second experimental day, a sample of blood was again taken with the subject in a fasting condition. This time the 5 gm. of ferric ammonium citrate were accompanied with 10 gm. of calcium lactate. This amount of calcium salt furnished approximately 1.5 gm. of calcium. Blood samples were taken throughout the day at the same intervals as on the previous experimental day and analyzed in the same manner.

The method recommended by Moore (48) for drawning blood samples for serum iron analysis was followed with a few modifications. A 20 gauge, 2 1/4 inch needle was slipped into the vein of the arm while the arm was hanging vertically to facilitate the flow of blood. As the blood flowed through the needle, it was collected in 15 cc. dry centrifuge tubes which had previously been chilled in ice water. In preleminary experiments it was found that less hemolysis occurred with chilled tubes than with the parafin coated ones suggested by Moore (48). Care was taken to keep the blood flowing in a steady stream down the side of the tube to prevent hemolysis caused by one drop falling on another. Three centrifuge tubes were filled to about the 10 cc. mark in this manner, and 2 cc. of blood, for the determination of hemoglobin and total iron, were collected in a specimen vial containing lithium citrate as the anti-coagulant.

The tubes of blood were centrifuged for 45 minutes at 2700 r.p.m. immediately after collection. It was found necessary to stop the centrifuge at the end of about 20 minutes and loosen the fibrin of the cell-

free clot from the edges of the tube with a fine pointed stirring rod. The clot would then centrifuge down forming a white rubbery layer between the serum and the red blood cells. The clear serum was pipetted from the three centrifuge tubes, combined and thoroughly mixed. If hemolysis was apparent or suspected in any of the tubes, the serum from that tube was not used.

The iron analyses of both the whole blood and serum were carried out on a micro scale. Preleminary analyses were preformed on quantities of serum varying from 0.1 cc. to 3 cc. to determine the smallest amount which could be depended upon to give checks within 5 per cent on duplicate or triplicate samples. Keeping the quantity of serum used in each analysis small was important because of the difficulty involved in obtaining large amounts of blood with only the pressure exerted in the veins and the gravitational force to draw the blood through the needle. In one subject who had a very short clotting time, the drawing of the blood was especially difficult.

A number of different procedures of analysis were tried before a satisfactory one for the small amount of serum available was found. The method of Breuer and Militzer (14) in which they digested the blood in H_2SO_4 without heating and then oxidized the sample with $KMnO_4$ and H_2O_2 was satisfactory with whole blood but did not completely digest the larger amount of serum used, and as a result a cloudiness appeared which interferred with reading the intensity of the color in the photelometer. This cloudiness was not removed either by filtering or centrifuging. Extraction of the color with iso amyl alcohol as recommended by Kennedy (36) tended to form an emulsion which also interferred with photometric determinations. Although the emulsion would break and the solution

become clear after the sample had stood for an hour or two, there was no certainty that the change in the photelometer reading was due entirely to the clearing of the solution and not to some fading of the color. As the photelometer is much more sensitive to faint colors than the colorimeter, it was found to be unnecessary to intensify the color by absorbing it with a solvent.

The method found to be most satisfactory combined suggestions from several different workers, (48, 14, 41). Triplicate samples of 3 cc. of serum each were pipetted into 10 cc. volumetric flasks. One cc. of concentrated H_2SO_4 was added to each sample and the two mixed thoroughly. The samples were digested on a steam heated hot plate, which maintained a temperature of from 85° to 90° C., for 24 hours or until they were reduced to one-half their original volume. The flasks were then placed on an electric hot plate and the temperature increased enough to produce gentle bubling of the digest mixture. An asbestos sheet was used on top of the hot plate to keep the flask from becoming so hot on the bottom that some of the material was blown out of the flask during the digestion and oxidation. When SO_2 fumes began to form, 60 per cent perchloric acid was added 3 or 4 drops at a time. Small funnels made from glass tubing were placed in the top of each flask at this time to prevent loss of any of the sample due to the spattering produced by the addition of the perchloric acid. When perchloric acid fumes ceased to appear after the first addition a few more drops of the oxidizing agent were added. This last process was repeated until all of the organic matter was oxidized and the solutions became clear and colorless. Any excess perchloric acid, which gave a yellow color to the solution, was removed by rapidly boiling the sample for a few minutes. Particles of charred organic matter which

adhered to the sides of the flasks were removed by washing them down with a drop or two of perchloric acid and then boiling the solution vigorously. Condensation of some of the acid fumes in the neck of the flask also helped to wash down the sides of the flask. The processes of digestion and oxidation usually required about 48 hours of constant heating. It was important during the early part of the digestion period to keep the temperature low enough to prevent popping out of the samples and at the same time hot enough that digestion took place. A temperature between 85° and 90° C. was found to be the best.

When oxidation was complete, the flasks were removed from the hot plate and allowed to cool for a few minutes. Seven cc. of water were added to each flask and mixed thoroughly with the acid digest. The solutions were cooled to room temperature under a stream of running water and 0.5 cc. of a saturated solution of potassium persulfate was added to each. The potassium persulfate served to keep the iron in an oxidized state and thus stabilized the color of the ferric thiocyanate compound. One cc. of 20 per cent potassium thiocyanate was added and enough water to bring the volume to the mark. The solutions were always at room temperature when made to volume. This solution was allowed to stand for at least one minute to develop the maximum color intensity which was found to be stable for at least 15 minutes. When the color was fully developed, the solution was placed in the micro cell of a Cenco Photelometer and the reading taken with the blue filter No. 554, 4.49 mm. The readings were converted to milligrams of iron by use of the standard calibration curve which had previously been prepared. The color was developed and read in only one sample at a time to eliminate variation in color due to the elapse of time. All of the water used in making up solutions and for rinsing

glassware was double distilled in glass.

The standard calibration curve for use in this experiment was constructed in the following manner: A standard iron solution as recommended by Breuer and Militzer (14) was prepared by dissolving 0.7 gm. of ferrous ammonium sulfate in 50 cc. of water; adding 50 cc. of 10 per cent H_2SO_4; then warming and adding 0.1 N potassium permanganate solution until oxidation was complete. This solution was transferred quantitatively to a liter volumetric flask and diluted to the mark with water. This primary standard contained 0.1 mg. of iron per cc. of solution. A secondary standard was prepared by diluting the primary standard 1 to 10. To make the photelometer readings, quantities of the secondary standard which supplied the desired amount of iron were pipetted into 10 cc. volumetric flasks. One cc. of concentrated H_2SO_4 was added and enough water to bring the volume to about 8.5 cc. The solutions were cooled and 0.5 cc. of potassium persulfate was added to each flask. The color was developed with 1 cc. of KCNS and the volume brought to the mark by the addition of a few drops of water. As in the unknown solutions, one minute was allowed to pass to assure maximum color development before the readings on the photelometer were taken.

Table II gives the photelometer readings for duplicate standard iron solutions covering the range within which all of the determinations in the experiment fell. The widest variation of readings on any duplicate standard solutions was 0.5 of a unit or 1.6 per cent which occurred at the 0.05 mg. level. This accuracy of the instrument was greater than could consistently be obtained on duplicate samples of the unknown.

A straight line relationship was found to exist between the log transmission and the concentration of the iron. In other words the lin-

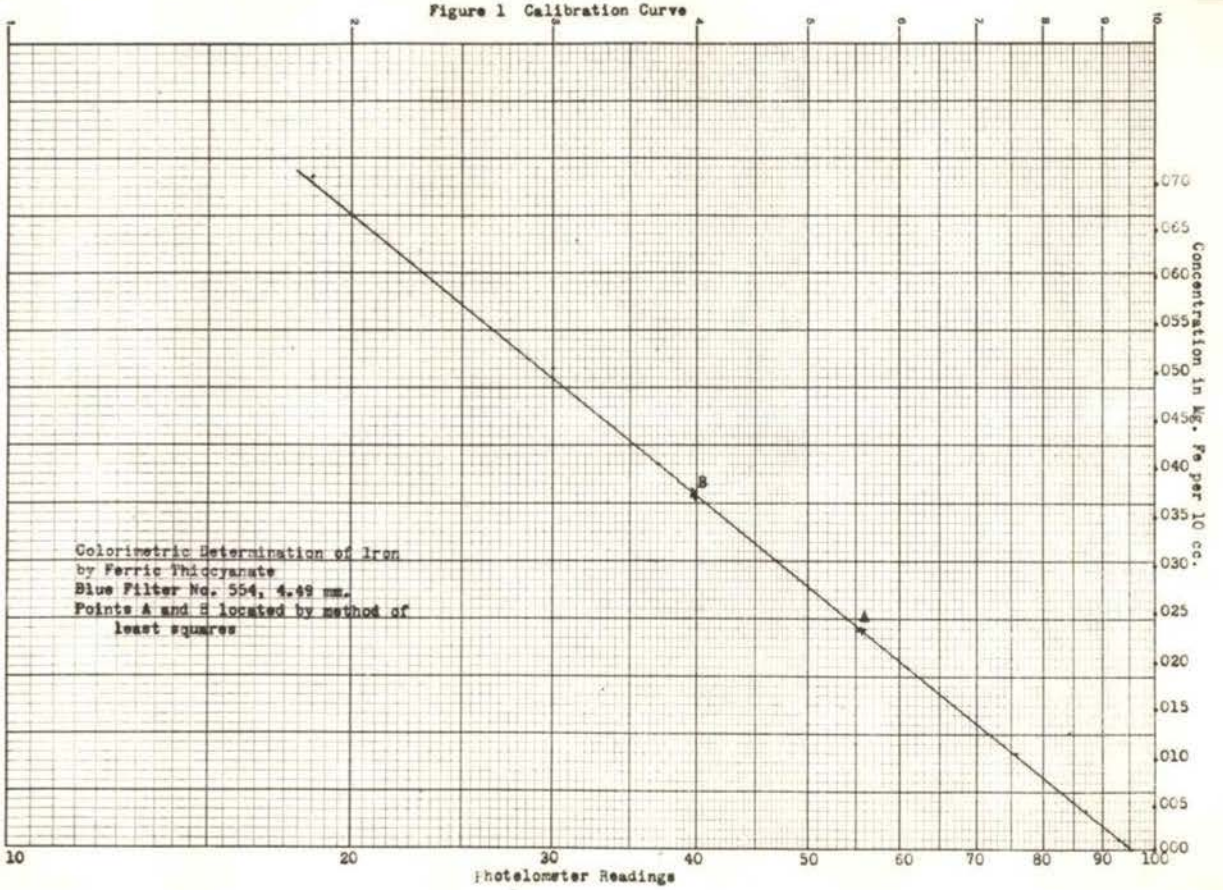
TABLE II

Photelometer Readings
for
Standard Iron Solutions

Mg. Fe in Standard	Readings*		
	1	2	Mean
0.0	95.0	95.1	95.0
0.0005	94.2	94.0	94.1
0.001	93.0	93.0	93.0
0.004	87.0	87.0	87.0
0.010	75.9	75.9	75.9
0.030	47.1	46.9	47.0
0.040	37.1	37.0	37.0
0.050	30.2	29.7	30.0
0.070	18.5	18.5	18.5

* Cenco Photelometer with Blue Filter No. 554, 4.49 mm.

Figure 1 Calibration Curve



ear regression follows the Bouger-Beer law as applied to the photelometer by the equation (15):

$$c = -\frac{1}{k}\log_{10}\left(\frac{P}{100}\right)$$

where c is the concentration of the iron in solution; P the photelometer scale reading or transmitted light; 100 the intensity of the incident light; and k the photelometer extinction coefficient.

The equation of the line for the observed points was derived by the method of least squares (68). The regression coefficient was determined by the formula:

$$r = \frac{V_{xy}}{V_x}$$

with r as the regression coefficient; V_{xy} as the covariance of the concentration, x, and the log transmission, y, and; V_x the variance of x. For this line r was calculated to be 10.1042 or, in other words, with every 0.01 mg. increase in the concentration of iron the log transmission decreased by 10.1042 divisions.

Since a logarithmic relationship exists between the photelometer readings and the iron concentration, calculations were simplified by plotting the calibration curve on semi-logarithmic paper. The known iron concentrations were plotted as ordinates on the equal division axis, X, and photelometer readings as abscissas on the logarithmic axis, Y. The calibration curve thus constructed is illustrated in Figure 1.

To calculate the amount of iron in the unknown solution from the calibration curve, the photelometer reading was located on the base line and followed from it in a vertical line to the point at which it crossed the calibration curve. A straight edge was layed horizontally through this point in such a manner that it crossed the "Mg. of Fe" scale on the

right. The value thus found represented the number of milligrams of iron in the 10 cc. volume. To convert this to 100 cc. of blood or serum as the case required, the value was divided by the number of cc. of sample used and multiplied by 100.

Total iron in whole blood was determined by the same method as that used for serum iron with the exception of the size of the sample. A 0.1 cc. sample was used for the whole blood instead of the 3 cc. sample of serum, since the high iron content made it possible to get checks within 5 per cent on this small volume. With samples of 0.1 cc. the readings of the photelometer for total blood iron fell within the limits of the same calibration curve as that used for serum.

The hemoglobin content of each of the blood samples was determined by the Newcomer (52) acid hematin method.

RESULTS AND DISCUSSION

The results of this experiment are presented in Tables III and IV. Because of the small number of subjects used and the variations in the results obtained, each subject is presented individually.

In all three subjects the iron in the blood serum increased following the ingestion of 5 grams of ferric ammonium citrate. From Table III it can be observed that following the initial rise during the first hour after administration of the iron salt, the value for the serum iron remained at approximately the same level throughout the six hours of the experimental period. The greatest increase in the iron content of the serum occurred in subject G.M. The amount of rise in the level of the serum iron of this subject was almost 1/3 greater than that of subject M.V. and 2/3 greater than that of subject E.C. However, it will be noted that the original level of the serum iron in the fasting condition is not the same for the three subjects; G.M. had the low value of 0.160 mg. of iron per 100 cc. of serum, E. C. the high value of 0.305 mg. and M.V. the intermediate value of 0.231. The differences in the levels of the serum iron of the three subjects were not as wide at the peak of absorption following the ingestion of the iron salt as they were in the fasting period. This seemed to indicate that the amount of iron absorbed may have been influenced by the level of the iron in the serum at the time the iron salt was administered.

The responses of the levels of the serum iron were not in agreement for the three subjects following the simultaneous administration of 5 gm. of ferric ammonium citrate and 10 gm. calcium lactate. In subject G.M. there was no absorption of iron as shown by the failure of the serum iron to increase. In fact, the iron level fell slightly below that of

TABLE III

Individual values for serum iron, total blood iron and hemoglobin at intervals following the oral administration of, I, 5 grams of Ferric ammonium citrate and, II, 5 grams of Ferric ammonium citrate plus 10 grams of Calcium lactate.

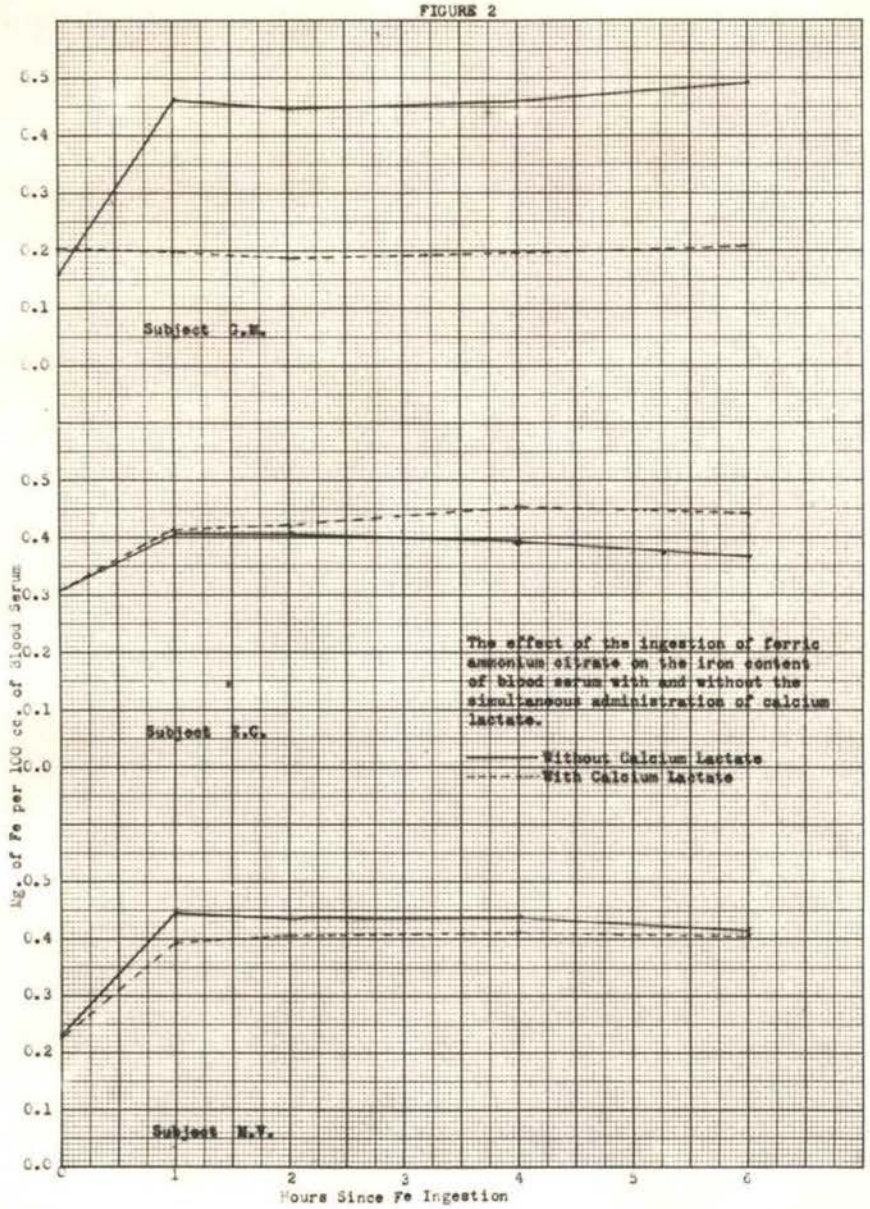
Subject	Determination of	Salts	Hours after administration of salts: 0	1	2	4	6
G.M.	Serum Iron (mg./100cc. serum)	I	0.160	0.460	0.446	0.460	0.490
		II	0.202	0.197	0.188	0.197	0.207
	Whole Blood Iron (mg./100cc.blood)	I	49.0	50.8	46.4	48.7	49.7
		II	43.6	49.2	48.6	49.8	--
	Hemoglobin (gm./100cc.blood)	I	14.6	14.0	13.7	13.8	13.7
		II	11.4	12.4	12.6	--	--
E.C.	Serum Iron (mg./100cc.serum)	I	0.305	0.403	0.402	0.393	0.368
		II	0.306	0.411	0.422	0.451	0.443
	Whole Blood Iron (mg./100cc.blood)	I	47.2	45.9	45.0	45.2	--
		II	47.5	44.2	44.1	46.1	42.2
	Hemoglobin (Gm./100cc.blood)	I	12.8	11.5	11.5	12.6	12.4
		II	12.3	11.6	11.4	11.7	11.8
M.V.	Serum Iron (mg./100cc.serum)	I	0.231	0.445	0.437	0.438	0.414
		II	0.228	0.396	0.405	0.411	0.405
	Whole Blood Iron (mg./100cc.blood)	I	47.1	44.1	46.2	46.0	46.2
		II	44.2	46.3	45.7	47.1	46.5
	Hemoglobin (gm./100cc.blood)	I	11.5	11.6	11.6	11.5	11.4
		II	12.1	11.3	11.0	11.2	11.0

TABLE IV

Deviations in the iron content of blood serum following the oral administration of, I, 5 grams of Ferric ammonium citrate and, II, 5 grams of Ferric ammonium citrate plus 10 grams of Calcium lactate from the values for serum iron of fasting subjects.

Subject	Serum Fe after fasting mg./100 cc. serum	Salts ingested	Deviation of serum Fe in mg. per 100 cc. from fasting value at intervals following ingestion of salts.			
			1 hr.	2 hr.	4 hr.	6 hr.
G.M.	0.160	I	0.300	0.286	0.300	0.330
	0.202	II	-0.005	-0.014	-0.005	0.005
E.C.	0.305	I	0.098	0.097	0.088	0.063
	0.306	II	0.105	0.116	0.145	0.137
M.V.	0.231	I	0.214	0.206	0.207	0.183
	0.228	II	0.168	0.177	0.133	0.177

FIGURE 2


Subject C.M.
Subject K.G.
Subject M.V.
The effect of the ingestion of ferric ammonium citrate on the iron content of blood serum with and without the simultaneous administration of calcium lactate.
Without Calcium Lactate
With Calcium Lactate
Mg. of Fe per 100 cc. of Blood Serum
Hours Since Fe Ingestion

the fasting blood, as can be seen by the negative deviation values in Table IV. In subject E.C. the serum iron rose to a higher level following the ingestion of both iron and calcium salts than it had with the iron alone. In subject M.V. the level of serum iron rose approximately 3/4 as high with calcium and iron together as it had when iron was given without the calcium.

Figure 2 illustrates, graphically, for both experimental days, the level of the serum iron during a number of hours after the administration of the salts.

Under the conditions of the experiment, the effect of calcium on the absorption of iron varied with the individual subject. The results indicate that comparatively large amounts of calcium may have a depressive effect on the absorption of iron in some cases and that the extent of the effect may depend in part on the original level of the serum iron. However before any definite conclusion can be drawn more subjects need to be studied and also other factors which may affect the problem. Some factors which may affect iron absorption are the pH of the gastro-intestinal tract (21, 51), the level of phosphorus and calcium in the blood of the subject (16) and the ratio of calcium and phosphorus in the diet preceeding the experimental days (4).

The ingestion of either the iron or the iron and calcium salts apparently had no effect that could be observed on the total iron content of the blood. It will be noted in Table III that the values for the total iron of whole blood varied slightly throughout the day but there was no consistent tendency in any specific direction. The small variation in the total iron content of blood following the absorption of iron is concealed by the high iron content of whole blood. Tripli-

cate analyses of the same blood sample may show as much variation in iron content within the range of experimental error as the difference in the iron present in samples taken before and after the absorption of the iron salts.

Variations in the hemoglobin values also failed to show any relationship to the ingestion of iron and calcium salts. In some instances the hemoglobin value fell about 1 gm. per 100 cc. of blood during the period between the taking of the first and second blood samples. However, this did not occur in all cases and there is no apparent explanation for this drop in the level of the hemoglobin.

In comparing the results obtained in this experiment with those reported by other workers, it may be stated that the amount of rise in serum iron following the ingestion of 5 gm. ferric ammonium citrated was practically the same as that obtained by Moore and coworkers (49). In the subject reported by Moore, et al, the absorption of the iron was more gradual and the peak value occurred at about the fourth hour, whereas in this study the peak of absorption occurred during the first hour and the serum retained this iron content throughout the six hours observed.

CONCLUSIONS AND SUMMARY

Three young women were used as subjects in a study on the effect of a large intake (10 gms.) of calcium lactate on the absorption of iron following the administration of a large dose (5 gms.) of ferric ammonium citrate. The criterion adopted for measuring the amount of iron absorbed was the change in the level of the serum iron from that of the fasting subject before the salts were administered, to that observed at intervals of 1, 2, 4, and 6 hours following the ingestion of the salts.

The gradient of the level of the serum iron during the absorption period on the first experimental day when only iron was administered tended to vary inversely with the initial level of serum iron. In the three subjects, G.M., E.C. and M.V., the greatest increases in the amount of iron present in the blood serum were from 0.160 to 0.490, 0.305 to 0.403, and 0.231 to 0.445, respectively.

When the calcium salt was administered with the iron salt on the second experimental day, the subjects responded in different ways. In one subject, G.M., the calcium lactate apparently prevented the absorption of the iron as the fasting value of the serum iron was 0.202 mg. per 100 cc. of serum and rose only to 0.207 mg. per 100 cc. In M.V. iron absorption occurred but to a lesser extent than when the iron alone was given, rising only from 0.228 mg. per 100 to 0.411 mg. per 100. In the third subject, E.C., the serum iron rose higher when both calcium and iron salts were ingested than when only iron was administered. The rise in this subject was from 0.306 mg. of iron per 100 cc. of serum to 0.451 mg. of iron.

The results indicate that in one instance the large intake of calcium lactate had a depressive effect on the absorption of the iron salts,

in another it may have had a slight effect and in a third no effect. The influence of the calcium was more marked in the subject with an initial low level of serum iron than in the one with a higher level.

More subjects should be observed and other factors investigated before more definite conclusions can be drawn.

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