A COMPARISON OF METHODS FOR THE STUDY OF PHYSIOLOGICAL AVAILABILITY OF FOOD IRON

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IRON AVAILABILITY

NAHAPETIAN

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AN ABSTRACT OF THE THESIS OF

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Title: A comparison of methods for the study of the physiological availability of food iron.

Iron is an essential micronutrient both for plants and animals. It has been found that iron deficiency anemia is a serious nutritional problem in the Middle East, even though dietary surveys have shown an apparently adequate iron intake. Thus it appears that the food iron in Middle Eastern dietaries is not well utilized. Information is needed on the physiological availability of iron in Middle Eastern foods, especially on those of plant origin which provide most of the dietary iron.

The purpose of the present study was to adapt the double isotope technique used in humans to rats for the study of the absorption of iron from various human foods. Two feeding methods were compared; using bread and purslane labeled with iron-55 as the test dietary doses and ferrous ascorbate labeled with iron-59 as the control test chemical. Moreover, preliminary experiments were undertaken before the main iron absorption studies, in order to ascertain whether or not the injected radioiron in plants is in a "physiological" form. Finally, in the last experiment, the "adapted" double isotope technique was used to study the effect of phytate on iron absorption and to investigate the physiological availability of hemoglobin iron in the rat.

Preliminary experiments with celery provided strong evidence that injected isotopic iron was in a "physiological" form, representative of the entire iron pool in the plant.

The results of feeding the test diets to anemic rats in metabolic cages indicated that there was no significant difference between the availability of food iron (bread and purslane) and ferrous ascorbate, which was fed by stomach tube. However, when both the test foods and ferrous ascorbate were given by stomach tube, the food iron was much less available than the inorganic iron. Finally, it was found that phytate inhibited iron absorption in the rat. Moreover, absolute absorption of hemoglobin iron was practically nil in all the rats under investigation, except in one. Statistical analysis showed that there was no significant difference among the relative iron absorption values of bread, purslane, and iron phytate, but relative iron absorption from hemoglobin was significantly less than all the other test diets.

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I. INTRODUCTION

It was believed in ancient times that iron was of heavenly origin. Anbar, meaning sky and fire, was one of the oldest names for iron (99). It has been said that anemia was recognized in ancient Greece, and treatment consisted of drinking water in which a sword had been allowed to rust (56).

Iron is an essential micronutrient for both plants and animals. It is a component of an electron transferring protein, ferredoxin, found in plants and bacteria (72, 77, 87). In the animal body, it does not exist in the free state, but is associated with several other compounds which are involved in oxidative processes and oxygen transport (12, p. 3). Iron is a component of two very important biocatalysts, hemoglobin and cell hemins (74, p. 372). Heme properties are similar to those of the iron atom. "Simple aqueous ferric ion has a catalytic activity expressed by the number 10^{-5}. If, however we surround that iron ion with a suitable organic grouping, it turns out that the catalytic ability of that iron has been enhanced by a factor of 1000. If we build the heme into a still more complex structure with a protein around it, we can increase the catalytic ability by several more powers of 10" (99, p. 5).

The normal adult body contains about 45 mg of iron per kilogram, 33 ± 5 mg of which are present in the form of hemoglobin, myoglobin and cell enzyme iron, and 12 ± 8 mg in the form of ferritin and

hemosiderin, as storage iron (46). It has been postulated that by controlling iron absorption, iron balance is maintained (52, 53, 82), enhanced absorption being found with increased need (27, 40, 46, 47, 57, 91, 92) as in the case of iron deficiency anemia. Moreover, there is diminished absorption with decreased need (13, 20, 46, 47, 78, 91). Thus, it is accepted that iron absorption through the intestinal tract is regulated, but so far as the nature of the regulatory mechanisms is concerned, there is no clear cut agreement.

Two assumptions had been held as part of all earlier hypotheses on the mechanism of control of iron absorption: (a) it was believed that the iron which entered the intestinal epithelial cells participated in the control of further iron absorption, and (b) all iron which entered the intestinal epithelial cells was absorbed into the blood (51, 60, 61). Later, it was found that the second assumption was not valid. It was postulated that iron in the epithelial mucosal cells might either be absorbed into blood or, might be desquamated into the intestinal lumen with the epithelial cells (25, 26). For movement of iron through the mucosal cells, a dual pathway was postulated and it was believed that ferritin was the form of iron which is slowly released (54). Increased amount of ferritin in the intestinal mucosa of guinea pigs was found 4 hours after feeding large amounts of iron (50). Results in later investigations were in agreement with the concept of a dual pathway for iron in intestinal mucosal cells. It was found that the quantity of iron put into a slowly released storage form was governed by the relationship between the size of administered dose to the capacity of

the rapid transport mechanism. For instance, a significant proportion of iron was turned into the stable form if the amount of iron given was large in relation to the capacity for rapid transport. However, the amount of iron put into stable form was relatively small, when the amount of administered iron was small in relation to the capacity of rapid transport. Thus, the amount of incorporated iron lost back into the intestinal lumen due to sloughing of the epithelial cells depended on the amount of iron fed (118).

More recently, it was postulated that when plasma iron is high, more iron is incorporated into ferritin bodies of the intestinal epithelial cells and there is inhibition of dietary iron absorption. In contrast, when plasma iron is low and dietary iron is deficient, mucosal iron concentration declines, less iron being incorporated into ferritin bodies. Flow is then recovered as iron is accepted into blood from the lumen (36). However, other recent investigations have indicated that there are pools, other than the hepatic or erythrocytic iron pools which control iron absorption. In an experiment with rats, iron absorption was doubled when rats were deprived of iron for 5 days by feeding them on an iron deficient diet (75). It is believed that since the present hypothesis of iron absorption is based on iron salt experiments, it is not adequate to explain absorption of all types of food iron such as hemoglobin (110).

Iron deficiency anemia is a serious nutritional problem in the Middle East, even though dietary surveys have shown an apparently adequate iron intake (29, 30, 33). Thus, it appears that the food

iron in Middle Eastern dietaries is not well utilized. Information is needed on the physiological availability of iron in Middle Eastern foods, especially on those of plant origin which provide most of the dietary iron.

The purpose of the present study was to adapt the double isotope technique used in humans to rats for the study of the absorption of iron from various human foods. If rat data correlate well with results of similar studies in humans, then the rat might serve as an inexpensive and convenient experimental animal for the valid assessment of human dietary iron sources. In this study, two feeding methods were compared and an attempt has been made to show contradicting results that may arise by using different methods of feeding. Moreover, a few preliminary experiments were undertaken before the main iron absorption studies, in order to ascertain whether or not the injected radioiron in one of the plants (celery) was found in a "physiological" form.

II. REVIEW OF LITERATURE

Physiological Availability of Iron

A relatively complete review of literature on iron availability studies has been presented earlier (45, pp. 5-8) and only the most recent studies will be reviewed here. Since in the previous review (45), the literature on hemoglobin iron absorption was not covered, studies on this subject will be dealt with in greater detail.

Hussain et al. used the double isotope technique with humans to study the availability of the iron in ferritin, hemoglobin and wheat. In iron deficient subjects it was found that, while ferritin and hemoglobin iron were more available than wheat iron, availability was less than that of iron ascorbate (70). In a recent investigation, hemoglobin regeneration rate was used as a criterion for the assessment of the physiological availability of iron in various foodstuffs. It was found that availability values for okra, chard, parsley, kidney bean, lentil, broad bean, decorticated sesame, parboiled wheat, and brown bread, were 25, 45, 48, 59, 67, 63, 67, 62, and 75 percent, respectively, relative to ferrous ascorbate (100 percent). The relative values for whole wheat and chick pea were above 80 percent. When individual foods were incorporated into prepared dishes, relative availability values for each dish reflected that of the individual components (33).

It is believed that dietary hemoglobin, as a source of iron, should not be underestimated, especially in countries where meat constitutes a major portion of the diet (28). Until 11 years ago, most of the investigations on iron availability were limited to iron salts, and it was believed that only ionic iron was absorbed (53). In 1955 it was shown that a significant quantity of iron was absorbed in humans, from test doses of hemoglobin (115); however, it was found that ascorbic acid did not increase percent absorption (23, 110), while it did enhance absorption of inorganic iron (17, 47, 67, 86, 91, 110).

It was postulated that hemoglobin iron was absorbed into the intestinal cells as heme or hemoglobin (23). This hypothesis was supported both by observations that inorganic iron was absorbed less efficiently from food than hemoglobin iron, and by investigations which proved that absorption of hemoglobin iron was not affected by iron binding compounds (7, 62, 71, 110, 116). Thus it was suggested that heme and ionized irons were being absorbed through two different pathways (116). However, experiments with albino rats failed to produce similar results; absorption of ferrous sulfate was 7 times that of iron from labeled hemoglobin. Bleeding enhanced ferrous sulfate absorption 3-fold, while it failed to increase that of hemoglobin. Moreover, when rats were fed hemoglobin as their major source of dietary iron for 8 weeks, they developed iron deficiency anemia (116). In another study, in which Wistar rats were used, iron deficient animals absorbed 4 to 8 times as much iron from hemoglobin as did normal ones, but there was much higher absorption from ferrous

sulfate than from hemoglobin in both normal and iron deficient animals (8). Thus, it was pointed out that they lacked the pathway for heme absorption, and that interpretation of the results of experiments on rats in terms of human physiology are subject to question (116). The same investigators, in more recent work, have found that apparently guinea pigs do not lack the pathway for heme absorption, and absorption of greater quantities of hemoglobin iron by guinea pigs, was stated to be the sole reason for the use of guinea pigs in their study rather than rats (28).

Methodology

Different methods used for iron absorption studies have been reviewed previously (12, p. 8, 45, p. 8). Hemoglobin regeneration has been used as a criterion for iron absorption in many previous studies, especially before radioisotope techniques were developed (9, 31, 33, 42, 43, 45, 48, 63, 64, 89, 101, 108, 111, 119). It is believed that hemoglobin regeneration rate can be used as a valid criterion for iron absorption, provided the following conditions are considered (16, p. 32):

1. The sole limiting factor for hemoglobin regeneration rate should be absorbed dietary iron, all of which must be utilized for hemoglobin synthesis. Thus the magnitude of the oral dose is of great importance; the optimal amount absorbed should not be greater than the optimal amount needed for erythropoiesis.

2. During the period studied, extra loss of iron such as that

due to bleeding and pregnancy must be excluded.

3. Experimental subjects or animals should not have high iron stores; otherwise it will be impossible to judge, whether the increased rate of hemoglobin regeneration was due to the iron stores or due to iron absorbed.

4. The blood volume must be known and it must be constant.

5. The same amount of iron must be used in different groups, when comparing availability of different dietary sources of iron.

6. Since the hemoglobin regeneration rate is faster at lower hemoglobin values, the initial level must be approximately the same in all the groups under investigation.

7. All experimental observations should be made before hemoglobin concentration becomes normal.

8. In the groups under study, age and sex distributions should be similar.

9. To minimize the effect of other unknown factors, the individuals should be appropriately randomized, especially according to hemoglobin level.

As can be seen from the foregoing conditions, a number of factors may affect the results obtained by using hemoglobin regeneration as the criterion for iron absorption. It is difficult to draw valid conclusions in comparing availability of iron from different sources if all the conditions are not considered critically, especially when materials studied by different authors are to be compared (16, p. 33).

The discovery of artificially induced radioactive isotopes in 1934 by Curie and Joliot (5) and the development of their easy preparation by cyclotron in 1936, opened a promising way for metabolic studies through use of "labeled" molecules. In 1939, Hahn et al. (57) initiated the use of radioiron for metabolic studies. They believed that radioactive isotopes behaved exactly "like their inactive replicas in body physiology". In their study using iron-59 (Fe^{59}), they found peak absorption in the small intestine 4 to 8 hours after feeding. Since 1939, most investigations on iron absorption have been conducted with radioiron (5, 16, 17, 18, 19, 27, 38, 40, 47, 58, 59, 60, 70, 71, 79, 90, 93, 94, 98, 105, 106, 110, 116).

The principle of radioiron experiments is as follows: the experimental subjects are either normal (71, 94, 110) or iron depleted due to natural causes, as with anemic individuals (40, 70, 71), or by artificial means, such as bleeding (59) and chemical treatment (27). The radioiron, in chemical form or incorporated in test diets, is fed after overnight fasting of the subjects. Blood samples are taken about 2 weeks after the last feeding for the determination of percent absorption. In earlier studies, Fe^{59} was counted with a Geiger-Muller counter which detects beta emissions. However, more efficient results have been attained by counting the gamma rays using crystal scintillation detectors (27, 47, 94).

For the comparative absorption of iron administered in different forms and under different conditions, double isotope techniques are the most reliable, since two different forms of iron can be compared in the same individual and within the same period. Thus,

factors such as anemia, age, sex, erythropoietic activity, blood volume changes etc. will not influence the experimental results, especially if an experimental design similar to that of Brise and Hallberg (16) is used. In earlier double isotope techniques, one isotope of iron (Fe^{55}) was administered orally and the other (Fe^{59}) was given intravenously. The direct measurement of the internal distribution from plasma components was made possible by the second isotope (98). However, in comparative studies of absorbability of different iron compounds, results obtained by this method will be affected by factors such as, age, sex, erythropoietic activity, etc. (19, p. 7).

According to the double isotope technique of Brise and Hallberg, equal doses of elemental iron are administered for 10 days after an overnight fast. Two radioisotopes of iron (Fe^{55} and Fe^{59}) are fed on alternate days during the 10 day period. Then, the mean absorption ratio is calculated from measurements of Fe^{55} and Fe^{59} activity in a blood sample drawn 2 weeks after the last oral dose (19, p. 8). Similar methods with minor differences have been used by later workers (70).

Dietary Factors Affecting Iron Availability

The effect of dietary factors on iron absorption has been discussed by a number of authors (31, 45, pp. 46-51, 65, 83, 104, 109). Many foods appear to aid the utilization of dietary iron by reducing it or by maintaining it in a reduced state. Ascorbic acid has been

found to be quite efficient in promoting iron absorption (17, 47, 67, 86, 91, 110), mainly due to its reducing action. Other factors which were found to enhance iron absorption are, succinic acid (18), citric acid (67) fat, and carbohydrate (105). In contrast, compounds such as phytate (4, 69, 84, 97, 100, 110), phosphate (76, 107), and certain other chelating agents (7, 71) are said to reduce intestinal absorption of iron, although there is still a question as to the effect of phytate (31, 100, 114) and phosphorus (24).

III. MATERIALS AND METHODS

Experimental Animals

The details of breeding and preparation of the anemic experimental animals were the same as those previously described (45, p. 14). Albino male or female rats of Sprague-Dawley strain (Animal Suppliers, London Ltd., England,) were used.

Feeding and Housing Equipment

For feeding the test doses of foods in the first experiment, plastic metabolic cages (Maryland Plastics Inc., New York, U.S.A.) were used (Figure 1). Otherwise, stainless steel cages (School of Engineering, American University of Beirut, Beirut, Lebanon,) were utilized throughout the experiments. Bardic feeding tubes (American University Hospital, Beirut, Lebanon) were modified for administering the test doses in experiments 2 and 3 (Figure 2).

Animal Diets

The low iron diet used was the one developed earlier (45, p. 15) with a minor modification in experiment 3, where sucrose was replaced with dextrose. The diet was composed of casein, iron free mineral

Figure 1. A metabolic cage which was used for feeding the test diets (bread and purslane) in experiment 1.

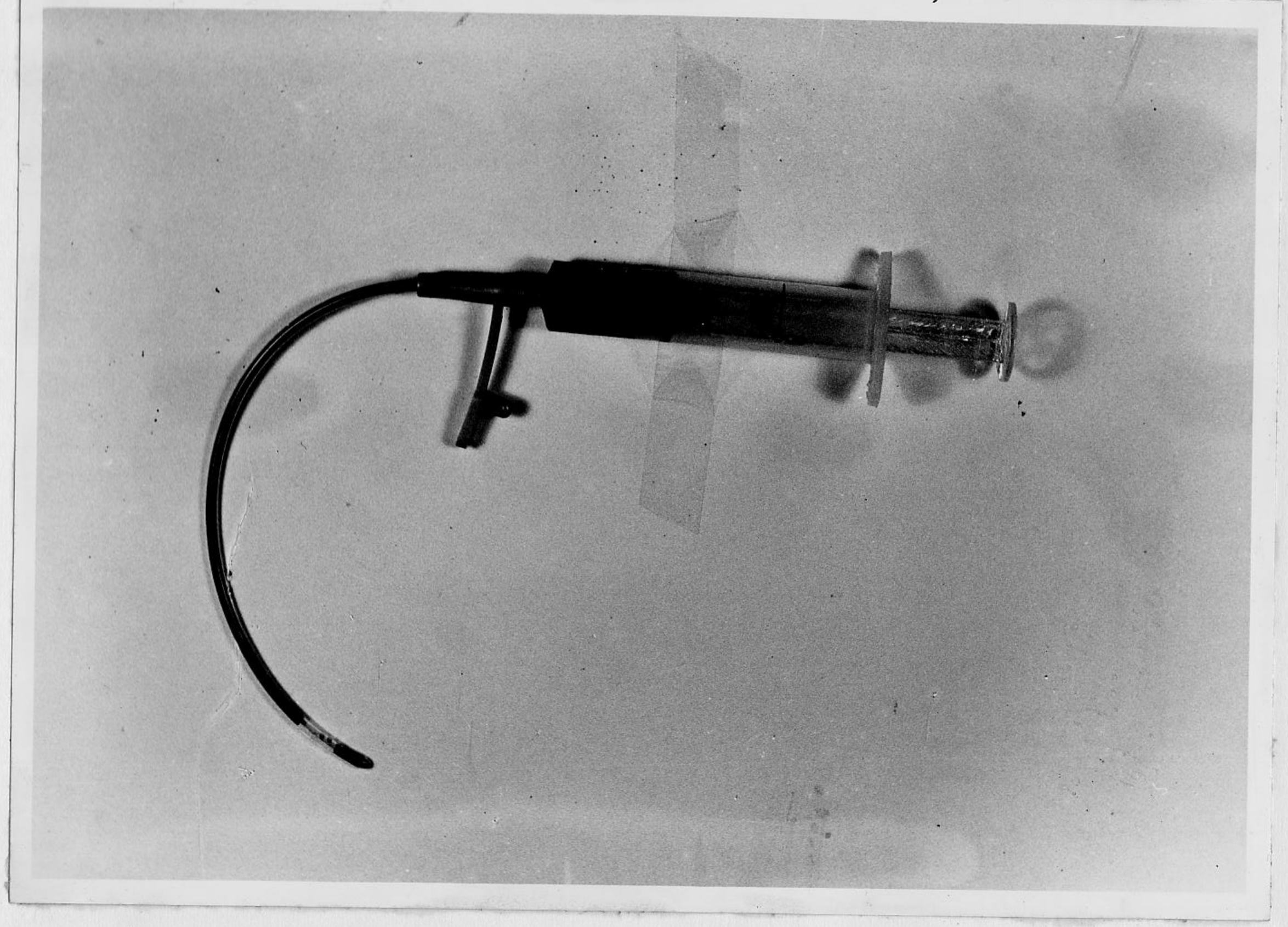

Figure 2. A stomach tube which was used for feeding the test diets (bread, purslane, iron phytate, and hemoglobin) and the control ferrous ascorbate chemical in experiments 2 and 3.

mixture, vitamin mixture, corn oil, and sucrose or dextrose (Appendix A). The iron content was 4 ± 3 micrograms per gram.

Preparation of Labeled Foods and Chemicals

Radioactive bread was prepared from wheat grown at the Agricultural Research and Education Center (AREC) of this university. When the seeds were at the milk stage, about 20 to 40 microcuries (uc) of $Fe^{55}Cl_3$ (Radiochemical Center, Amersham, England) were administered to each plant by stem injection. The seeds were allowed to mature naturally at which time they were harvested and dried at 70^{o} Centigrade (C) for 24 hours. The wheat was ground to a fine flour, which was then made into Arabic-type bread[1] by a method described elsewhere (39, pp. 12-13). The bread used for the first experiment was dried at 70^{o}C for 24 hours, ground and fed to the rats in a powdered form in metabolic cages. For the second experiment, the baked bread was fried in mazola oil, ground and suspended in water; the doses were administered by feeding tube to the rats. The specific activity of bread was about 13 uc per mg iron.

Mature plants of purslane (Portulaca oleracea) and celery (Apium graveolens var. botrytis) were transplanted from soil into plastic pots. After 2 days, about 20 uc of radioiron were administered to each plant by stem injection. One week later, the purslane was harvested, dried at 70^{o}C for 24 hours and ground to a powder, which was administered as such suspended in water. The specific activity of purslane was 1 uc per mg iron. The celery was used in autoradiographic

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[1] Made from whole grain (un-milled) wheat.

and other studies in an attempt to describe the form of injected iron in the plant.

Labeled hemoglobin was prepared using a modified technique previously described by Hussain et al. (70). Six intravenous injections of iron-55 (ferric salt) were administered under light ether anesthesia to mature anemic rats, over a period of 6 days, providing each animal with a total dose of 600 uc of radioactivity. Fourteen days after the last injection, the rats were anesthetized with chloroform and blood was obtained by cardiac puncture. The radioactive red cells were washed in saline solution and after repeated freezing and thawing, the stroma fraction was removed by centrifugation; the supernatant hemoglobin solution was stored at -10°C until it was used for feeding. The specific activity was about 8 uc per mg iron.

Radioactive iron phytate was prepared by dissolving excess sodium phytate with about 500 uc of $Fe^{55}Cl_3$ in 5 percent trichloroacetic acid (TCA) solution. The solution was heated for 30 minutes in a boiling water bath, and after centrifugation, the precipitate was washed with distilled water, diluted with dextrose, and freeze-dried. The dehydrated mixture was ground to a fine powder after which it was suspended in water for administration by stomach tube to the animals. The specific activity of iron phytate was about 60 uc per mg iron.

Labeled ferrous ascorbate for the control doses was prepared as follows: four mg of iron as ferrous sulfate ($FeSO_4.7H_2O$), 1.0 g ascorbic acid, and 50 uc iron-59 as $Fe^{59}Cl_3$ were mixed in a 50 ml

volumetric flask. The solution was made to volume with 0.02 N HCl and stored at 4°C until needed. The specific activity of the ascorbate was about 12 uc per mg iron. The pH of the solution was 2.2, which was within the range recommended for maximum iron absorption (79).

Determination of Form of Iron in Celery

A series of experiments was performed with a green plant (celery), to determine whether or not injected iron in the plant was in a "physiological" form, i.e., bound to large molecules. Celery was used in this investigation, because of its availability and because of the ease in labeling by stem injection. Moreover, each plant could be utilized over a long period of time, since new shoots grew quickly following each harvest. The plants were transplanted from soil into pots containing soil. They were irrigated twice a week with Hogland's nutrients medium (66, p. 85); otherwise tap water was used throughout. Two days after transplanting, about 20 uc of Fe^{59} as $Fe^{59}Cl_3$ were administered to each plant by stem injection; after 4 days the plants were harvested. The fresh leaves were frozen with liquid air and crushed in a precooled mortar. The crushed plant material was squeezed through 4 layers of cheesecloth after which the filterate was centrifuged at 3000 revolutions per minute (rpm) for 15 minutes; the supernatant was then dialyzed for 30 hours in 2 liter portions of distilled water; the water was changed 5 times during the dialyzing period. The dialyzate was concentrated by flash evaporation and its specific activity together with that of the material left inside the

dialysis bag were determined using a well-type crystal scintillation counter (Baird-Atomic Inc., Model 135, Cambridge, Mass., U.S.A.).

Autoradiography of Radioactive Celery

In an attempt to find the distribution of radioiron injected into the celery, about 100 uc of Fe^{59} as $Fe^{59}Cl_3$ were administered to a single plant; after 4 days, one shoot of the treated celery was prepared for autoradiography by modification of a method suggested by Branton and Jacobson (14). An entire shoot was placed in a plastic transparent file, which was pressed between a thick felt base and a weighted metal tray. The shoot was frozen within seconds by pouring liquid air into the tray. Following freezing, the shoot was transferred into a cold X-ray cassette (24 X 30 cm) in which a Kodak medical X-ray film (Royal blue 24 X 30 cm) was enclosed. After 10 days of exposure at $-10^{o}C$ the film was developed with Kodak developer (DA-19b) in a dark-room equipped with a Kodak safety lamp (6B filter). After development, the films were rinsed with tap water and fixed with Kodak unifix solution.

Analytical Methods

Hemoglobin and iron determinations were carried out as described earlier (45, p. 17). Phytate was determined by the method of Lange et al. (80).

Determination of Iron Absorption

For the first experiment, a modified method similar to that described previously for humans was used (70). The rats were randomized in groups according to blood hemoglobin levels. After overnight fasting, they were fed the test diets (labeled bread and purslane) in metabolic cages (Figure 1). The test doses of bread and purslane, provided 3 and 0.3 uc respectively of iron-55 and 0.2 mg of elemental iron. Similar doses of iron have been used for iron absorption studies in rats (75). Earlier investigations have shown that about 60 to 80 percent of total iron absorption takes place within the first 2 hours in the rat (118). Thus no food was allowed for 3 hours after the test feeding, so that no dietary factor other than those present in the test doses would influence iron absorption. After a second overnight fast, the animals were dosed under light ether anesthesia with iron ascorbate (0.2 mg of iron and 1 uc of radioactivity). After 3 hours, during which no food was allowed, the rats were put on a commercially available laboratory animal food (Vitasni Feed Company, Beirut, Lebanon) for two weeks.

On the 14th day, the rats were weighed and sacrificed by chloroform and blood samples were obtained by cardiac puncture. These were ashed according to the method of Vosburgh et al. (113). After weighing the blood samples accurately in small porcelain crucibles, 1 or 2 ml of concentrated (conc.) nitric acid were added for each 5 gm of blood to prevent the possible loss of iron during ashing. Following the drying of the blood samples at 80°C for 24 hours, they were ashed

in a muffle furnace at 400-500°C for 24 hours. There was often a small amount of residual carbon subsequent to ashing, which was destroyed by moistening the ash with a few drops of conc. nitric acid, drying the residue on a hot plate, and then returning the crucibles to the hot muffle furnace for an additional 30 minutes. When ashing was completed, each ashed sample was cooled to room temperature and dissolved in 1 ml of conc. hydrochloric acid.

Each sample was then transferred quantitatively to a 10 ml volumetric flask which was made to volume with distilled water. One ml of each diluted solution was spotted within a 3 X 4 cm area on a strip of Whatman No. 1 chromatographic paper, which was then dried and transferred into a counting vial filled with the scintillation solution (Appendix B). After adjusting the proper counter settings (Appendix C) on a Packard Tri-Carb Scintillation Spectrometer (Packard Instrument Company, Inc., Model 3003, LaGrange, Illinois, U.S.A.) the amount of Fe^{55} and Fe^{59} in each sample was determined as counts per minute (cpm).

About one gram of each dietary test dose was weighed accurately in a small porcelain crucible, ashed, spotted and counted in the same manner as the blood samples. The specific activities of the test diets were determined both before the start of the main experiment and after the experimental animals were sacrificed. Dietary samples were ashed with blood samples in the same muffle furnace to minimize possible error due to fluctuating ashing temperatures (49, 113).

The total blood weight of the rat was presumed to be 5 percent of the body weight (27). Absolute and relative iron absorptions were

calculated according to the following formulae:

1. Absolute iron absorbed (percent) = $\frac{5 \times \text{body weight} \times \text{cpm per gram blood}}{\text{Total cpm given in test dose}}$

2. Relative iron absorbed (percent) = $\frac{\text{Absolute iron-55 absorbed}}{\text{Absolute iron-59 absorbed}} \times 100$

IV. RESULTS AND DISCUSSION

The Chemical Form of Iron in Celery

In order to determine whether the injected iron in celery was found in a "physiological" form, a few plants were injected with iron-59. The plants were then harvested and homogenized and the plant extract dialyzed against distilled water. It is evident, from the data in Table 1, that nearly all the radioactivity (98 percent) remained inside the dialysis bag with only 2 percent passing into the dialyzate. The results of the present work are in agreement with the fact that most of the iron in plants is bound to organic compounds that cannot pass through the pores of a dialyzing membrane because of their size. In 1941, Leibich (11, p. 259) reported that about 82 percent of spinach leaf iron was present in the chloroplasts, and four-fifths of this iron was found in organic combinations.

Table 1. Percent radioactivity of celery extract remaining or lost through the dialyzate after 30 hours of dialysis against distilled water.

	Percent radioactivity
Remaining in the dialysis bag	98
Lost with the dialyzate	2

Location of Iron-59 in Celery Shoots

An attempt was made to find the location of iron-59 in celery shoots, by autoradiography of the radioactive plants. One of the autoradiographs is shown in figure 3. It appeared that most of the radioactivity was concentrated at the end of the stem, which in the following experiments was found to be an artifact, resulting from the escaping of the plant fluid through the veins when the shoots were pressed between the blotters. However, a higher amount of iron-59 appeared to be concentrated in the vascular tissue in agreement with previous workers (6).

When the plant shoots were frozen quickly with liquid air and then used for autoradiography, no artifact appeared in the autoradiographs (Figure 4). By instantaneous freezing, the iron was fixed in the shoots at its natural location. Thus, a more realistic picture of its location in the celery shoots was obtained. As it is evident from figure 4, the radioiron was spread evenly throughout the entire shoot. Contrary to the previous autoradiograph (Figure 3), the radioactivity did not appear to be concentrated in the veins. For comparison, a single plant of celery was injected with iodine-131[1] and after quick freezing it was autoradiographed (Figure 5). Iodine-131 was used because it is known to be found in certain plants in a free inorganic state, not combined with organic compounds (32, 73). By

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1. Obtained from the Radiochemical Center, Amersham, England.

Figure 3. Autoradiograph of a celery shoot which was injected with iron-59 and was processed without freezing (10-day exposure to film).

Figure 4. Autoradiograph of a celery shoot, which was injected with iron-59 and frozen before processing (10-day exposure to film).

Figure 5. Autoradiograph of a celery shoot, which was injected with iodine-131 and frozen before processing (10-day exposure to film).

comparison of figures 4 and 5, it is evident that the iron-59 is not concentrated in the vascular tissue of celery, as much as iodine-131.

The autoradiographs showed that the injected iron was evenly distributed throughout the celery, providing evidence for its "physiological" localization in the healthy plants.

The stem-injection technique for labeling plants with radioisotopes was first used by Levy in 1939 (81). In a recent investigation, iron-59 incorporation experiments were conducted on corn (Zea mays), wheat (Triticum vulgare), and beans (Vicia faba). It was found that, when iron-59 was administered by stem injection, the isotope was incorporated in a physiological form (120). The results presented here (Table 1 and Figure 4), together with the findings of other workers, provide strong evidence that injected isotopic iron is found in a "physiological" form, representative of the entire iron pool in the plant.

Iron Absorption in the Rat

Experiments 1 and 2: Since there are wide variations in the magnitude of iron absorption among individual rats due to a number of factors which were discussed in the introduction, double isotope absorption studies were performed on each rat. Thus, each of the experimental animals served as its own control. In experiments 1 and 2, the same test diets were fed to the rats by different feeding practices. The results are given in tables 2 and 3, and are illustrated in figures 6 and 7. The doses of elemental iron were kept to either 0.2 mg or

Table 2. Absorption of bread (Fe^{55}) and ferrous ascorbate (Fe^{59}) test doses in anemic rats.

Rat Number	Hemoglobin concentration	Bread iron absorption	Ferrous ascorbate iron absorption	Relative iron absorption Fe^{55}/Fe^{59}
	g Percent	Percent	Percent	Percent
		Experiment 1		
1	5.90	64.84	63.00	102.92
2	6.40	72.51	90.93	79.74
3	7.17	55.41	52.95	104.65
4	-	69.80	75.09	93.00
5	6.43	66.02	51.48	128.24
6	6.70	61.82	33.85	182.63
7	7.33	52.78	54.99	95.98
Mean ± S.E.[x]	6.66 ± 0.09	63.32 ± 2.73	60.33 ± 6.95	112.45 ± 12.90
		Experiment 2		
1	4.34	29.49	63.27	46.60
2	4.84	32.55	79.83	40.77
3	5.34	30.43	77.37	39.33
4	5.67	30.61	71.69	42.69
5	5.67	34.53	70.48	48.99
6	6.10	26.60	73.33	36.27
Mean ± S.E.	5.33 ± 0.11	30.70 ± 1.06	72.66 ± 2.37	41.46 ± 1.90

Experiment 1. Experimental diets were fed in metabolic cages. Both bread and ferrous-ascorbate test doses provided 0.2 mg of iron.

Experiment 2. Experimental diets were fed with stomach tubes. Bread and ferrous-ascorbate test doses provided 0.02 and 0.03 mg of iron respectively.

[x] Standard error.

Table 3. Absorption of purslane (Fe^{55}) and ferrous ascorbate (Fe^{59}) test doses in anemic rats.

Rat number	Hemoglobin concentration	Purslane iron absorption	Ferrous ascorbate iron absorption	Relative iron absorption Fe^{55}/Fe^{59}
	g Percent	Percent	Percent	Percent
		Experiment 1		
1	5.87	79.58	56.96	139.71
2	6.13	79.95	58.51	136.67
3	7.33	72.95	42.95	169.85
4	-	80.23	50.78	158.00
5	5.87	75.66	53.59	141.18
6	5.93	67.85	42.77	158.64
7	7.33	66.66	41.96	158.87
Mean ± S.E.	6.69 ± 0.12	74.70 ± 2.17	49.65 ± 2.68	151.85 ± 4.76
		Experiment 2		
1	5.00	16.39	53.58	30.58
2	5.34	30.85	70.83	43.55
3	5.67	39.69	66.03	60.10
4	5.84	30.22	75.88	39.82
5	6.00	22.00	58.36	37.69
6	7.67	29.67	71.70	41.38
Mean ± S.E.	5.92 ± 0.16	28.14 ± 2.28	66.06 ± 3.49	42.19 ± 4.00

Experiment 1. Experimental diets were fed in metabolic cages. Both purslane and ferrous-ascorbate test doses provided 0.2 mg of iron.

Experiment 2. Experimental diets were fed with stomach tubes. Purslane and ferrous-ascorbate test doses provided 0.07 and 0.03 mg of iron respectively.

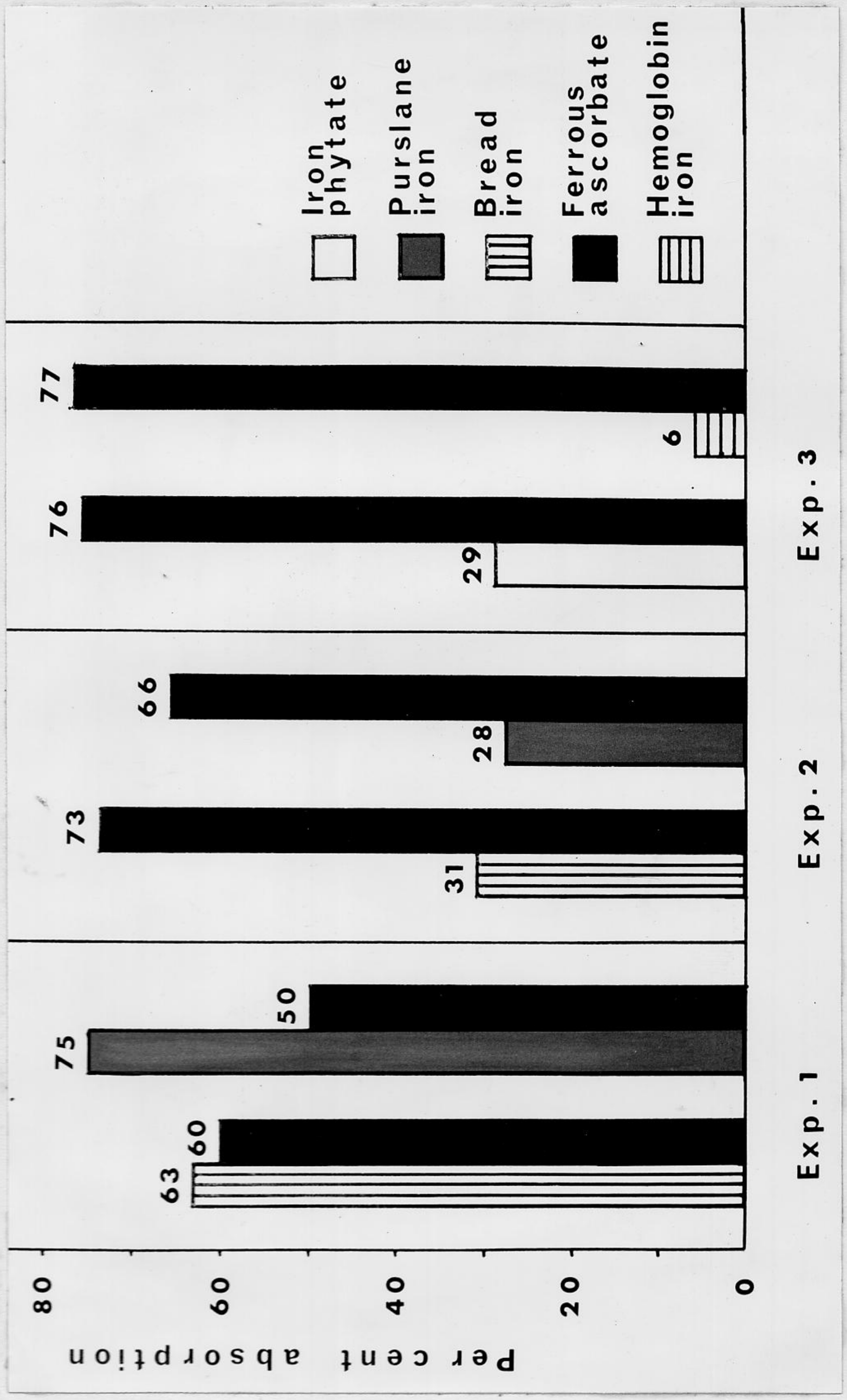

Figure 6. Absolute iron absorption from experimental foods, (bread, purslane, and hemoglobin), iron phytate, and ferrous ascorbate.

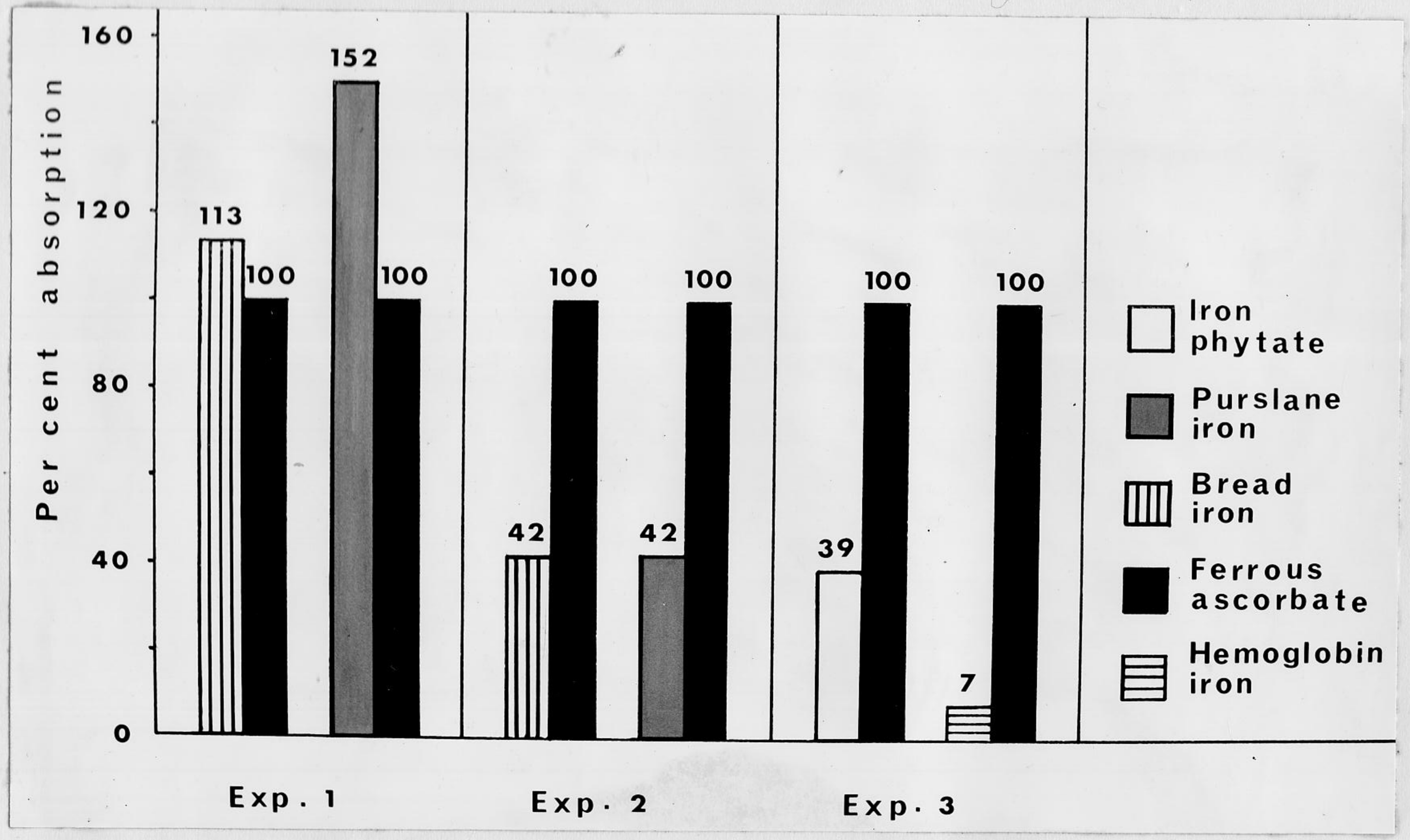

Figure 7. Relative iron absorption from experimental foods (bread, purslane, and hemoglobin), iron phytate, and ferrous ascorbate.

less, since larger doses exceed physiological limits.

The average initial hemoglobin concentrations in experiments 1 and 2 ranged between 5.30 to 6.66 g percent. In both experiments, the foodstuffs tested were bread and purslane. These were fed to the rats either in metabolic cages (Experiment 1) or by stomach tube (Experiment 2). The control doses of ferrous ascorbate were given by stomach tube.

It is evident (Table 2), from the results of the first experiment, that average absolute bread iron absorption (63.32 ± 2.73) was approximately as much as that of ferrous ascorbate (60.33 ± 6.95). Statistical analysis showed no significant difference between the two absorption values (Table 4).

These results were contrary to those of other workers (33, 34, 70) who found low absorption of bread iron. It was postulated that, since the radioactive bread was not fed in the same manner as the ferrous ascorbate control diet, the contradictory absorption values resulted. Whereas it took each rat an average of 24 hours to finish eating the test dose of bread, it took less than a minute to feed the entire control dose of ferrous ascorbate with a syringe. Thus, even though both the control and experimental diets provided the same amount of iron, they were not supplied to the gastric tract at the same rate.

When the bread was fed with a stomach tube (Experiment 2) the average absolute absorption value (30.70 ± 1.06 percent) was significantly less than that of ferrous ascorbate (72.66 ± 2.37 percent). The latter results agree with limited work on humans. In one investigation, it was found that anemic human subjects absorbed

Table 4. Significance of the t-tests between the absolute values for iron absorption in the test diets (bread, purslane, iron phytate, and hemoglobin) and iron ascorbate in experiments 1, 2, and 3.

Diets	Experiment	Significance
Bread vs ferrous ascorbate	1	NS[x]
Bread vs ferrous ascorbate	2	$P < 0.01$
Purslane vs ferrous ascorbate	1	$P < 0.01$
Purslane vs ferrous ascorbate	2	$P < 0.01$
Iron phytate vs ferrous ascorbate	3	$P < 0.01$
Hemoglobin vs ferrous ascorbate	3	$P < 0.01$

[x] Not significant.

7.8 ± 4.12 percent of wheat iron, while 44.2 ± 12.1 percent of ferrous ascorbate iron was absorbed (70). More recent results have shown 11.1 percent absorption of bread iron compared to 33.0 percent absorption from ferrous ascorbate (34).

The results obtained by feeding purslane test diets in metabolic cages (Experiment 1) or by stomach tube (Experiment 2) were similar to the contradictory results of bread in the two experiments. Average absolute percent absorption of purslane iron in the first experiment (Table 3 and Figure 6) was 74.70 ± 2.17 as compared to 49.65 ± 2.68 percent absorption from ferrous ascorbate. However, when both purslane and the control test diet were fed with a stomach tube, absolute percent absorption from purslane (28.14 ± 2.28) was significantly less than that from ferrous ascorbate (66.06 ± 3.49).

The results of relative absorption of bread and purslane iron are summarized in tables 2 and 3 and are illustrated in figure 7. The average relative absorption of bread iron (112.45 ± 12.90) and purslane iron (151.85 ± 4.76) in the first experiment were significantly more than the average relative iron absorption from bread (41.46 ± 1.90) and purslane (42.19 ± 4.00) in the second experiment. Thus, by using two different methods of feeding, relative absorption of iron from the same dietary sources differed significantly (Table 5).

Since the results obtained by following the procedure in the second experiment agree with available human data, and since similar methods of feeding the test foods and control test chemicals were thought to be more logical, it was concluded that feeding by stomach tube should be used in testing the physiological availability of

Table 5. Significance of the t-tests between the relative values for iron absorption in the test diets (bread, purslane, iron phytate and hemoglobin) in experiments 1, 2, and 3.

Diets	Between experiments	Significance
Bread vs bread	1 & 2	$P < 0.01$
Purslane vs purslane	1 & 2	$P < 0.01$
Bread vs purslane	2 & 2	NS[x]
Bread vs iron phytate	2 & 3	NS
Bread vs hemoglobin	2 & 3	$P < 0.01$
Hemoglobin vs purslane	3 & 2	$P < 0.01$
Hemoglobin vs iron phytate	3 & 3	$P < 0.01$
Iron phytate vs purslane	3 & 2	NS

[x] Not significant

dietary iron when rats are used as the experimental animals. By using the second experimental method of feeding, the main sources of error on comparative iron absorption studies were minimized. The repeated administration of both control and test diets, reduced the variation in absorption and internal distribution of absorbed iron. However, there are still three sources of error: (a) analytical errors, (b) errors in the administration of iron doses, and (c) variation in absorption and distribution of absorbed iron; the latter error can be reduced by more iron doses over a longer period of time (19).

The standard errors for relative absorption values for bread and purslane iron in experiment 1 were 12.90 and 4.76 respectively, while in the second experiment they were 1.90 for bread and 4.00 for purslane iron. These values show that more consistent results were obtained by the second method.

As was demonstrated, feeding practices affected the results obtained and it is difficult to draw valid conclusions in comparing availability of iron from different compounds, even when double isotope techniques are used, unless all the test diets are fed in the same manner. The considerations of feeding practices are critical especially when foodstuffs studied by different authors have to be compared.

Experiment 3: The purpose of the third experiment was to use the technique from experiment 2 to study the effect of phytate on iron absorption and to investigate the physiological availability of hemoglobin iron in the rat. The details for the preparation of

labeled hemoglobin and iron phytate were discussed earlier.

Even though the rats were kept on a low iron diet for 4 months, hemoglobin concentration levels were not low at the start of the experiment (Tables 6 and 7). However, it seemed evident from the range of absolute percent absorption values for ferrous ascorbate (46.77 - 104.93), that either (a) iron stores were depleted, or (b) an unknown metabolic pool of iron,other than that affected by hemoglobin level and body iron stores, was triggered for higher iron absorption when the rats were fed on an iron deficient diet. It is known that iron absorption in the rat increases with dietary iron deprivation (75).

The results of experiment 3 are given in tables 6 and 7 and are illustrated in figures 6 and 7. The average absolute absorption of iron from iron phytate (28.88 $\pm$ 3.12) was significantly less than that from ferrous ascorbate (76.08 $\pm$ 9.14). These results are in agreement with those of some investigators (4, 69, 84, 97, 100, 110); however, they are in conflict with those of others (31, 114, 45, p. 49). The controversy appears to be due to differences in experimental methodology and the form of phytate and iron in the test diets. For instance, Cowan et al. (31) used a basal low iron diet which was supplemented with ferrous sulfate and sodium phytate, while in the present study synthetic iron phytate was used. Further studies are necessary along this line to clarify the controversy present in the literature.

Absolute absorption of hemoglobin iron was practically nil in all the rats under investigation, except in one which absorbed about

Table 6. Absorption of iron phytate (Fe^{55}) and ferrous ascorbate (Fe^{59}) test doses in rats[x].

Rat number	Hemoglobin concentration	Iron phytate iron absorption	Ferrous ascorbate iron absorption	Relative absorption Fe^{55}/Fe^{59}
	g Percent	Percent	Percent	Percent
1	17.67	25.48	49.33	51.53
2	16.00	25.38	57.62	44.05
3	14.00	26.62	93.54	28.46
4	13.67	25.95	87.16	29.77
5	10.00	44.41	104.93	42.32
6	-	25.51	63.88	39.93
Mean ± S.E.	14.27 ± 1.16	28.88 ± 3.12	76.08 ± 9.14	39.34 ± 3.59

[x] Experiment 3. Prior to this experiment the rats were fed on a low iron diet for 4 months. Daily iron (0.08 mg) was provided either from iron phytate (0.11 mg phytate) or ferrous ascorbate, and the doses were given by stomach tubes.

Table 7. Absorption of hemoglobin (Fe^{55}) and ferrous ascorbate (Fe^{59}) test doses in rats[x].

Rat number	Hemoglobin concentration	Hemoglobin iron absorption	Ferrous ascorbate iron absorption	Relative absorption Fe^{55}/Fe^{59}
	g Percent	Percent	Percent	Percent
1	20.67	0.00	46.77	0.00
2	13.67	0.00	73.10	0.00
3	12.00	0.00	84.07	0.00
4	12.00	0.00	95.63	0.00
5	10.67	24.42	83.95	32.66
Mean ± S.E.	13.80 ± 0.80	5.48 ± 5.48	76.70 ± 8.34	6.53 ± 6.53

[x] Experiment 3. Prior to this experiment the rats were fed on a low iron diet for 4 months. Daily iron (0.08 mg) was provided either from hemoglobin or ferrous-ascorbate, and the doses were given by stomach tubes.

24.42 percent of the administered dose (Table 7). However, the average absolute iron absorption from ferrous ascorbate was 76.70 ± 8.34 percent. Statistical analysis showed that iron absorption from hemoglobin was significantly less than that from ferrous ascorbate (Table 4). The absorption values were in agreement with the results of other investigations on rats (8, 116), but they did not agree with human data. In 1955, Walsh et al. (115) showed that a significant quantity of hemoglobin iron was absorbed in humans. More recently, similar results were obtained by other investigators, with average hemoglobin iron absorption values of 21.4 ± 4.20 percent in anemic subjects compared with 44.2 ± 19.1 percent absorption of ferrous ascorbate (70). However, since one of the rat values was similar to human data, more work is needed for a valid conclusion. As pointed out earlier (116), the rat may lack the pathway for heme absorption, and interpretation of results of experiments on rats in terms of human physiology are subject to question.

Comparison of Relative Iron Absorptions of Experimental Test Diets

Relative iron absorption values for bread, purslane, hemoglobin and iron phytate are presented in tables 2, 3, 6, and 7 respectively. A summary of the relative absorption values of all test diets is illustrated in figure 7. Statistical analysis (Table 5) showed that there was no significant difference among the relative iron absorption values of bread, purslane, and iron phytate. However, relative iron

absorption from hemoglobin was significantly less than all the other test diets.

V. SUMMARY AND CONCLUSIONS

The purpose of the present study was to adapt the double isotope technique used in humans to rats for the study of the "physiological" availability of iron from various human foods. Two preliminary and three main absorption experiments were performed to investigate:

1. The location of iron injected in celery and whether it is in a "physiological" form.

2. The effect of two feeding methods of the test materials: in metabolic cages or by stomach tube.

3. The physiological availability of iron from food sources (bread, purslane, hemoglobin) and from iron phytate.

Preliminary experiments provided evidence that injected isotopic iron was found in a "physiological" form.

In the main experiments 1 and 2, contradicting results were obtained by using two methods of feeding (in metabolic cages or by stomach tube) for the test doses of bread and purslane. Data similar to those from human experiments were obtained with the latter method.

In experiment 3, it was found that phytic acid inhibited iron absorption and that absolute absorption of hemoglobin iron was practically nil in all the rats under investigation except in one.

It was concluded that the double isotope method of studying iron absorption might be used to obtain valid absorption data on

"available" iron in human foods, provided the stomach tube technique is used for feeding of both control and test diets. However, unlike human beings since the rat might lack the pathway for heme absorption, and interpretation of results of experiments on rats in terms of human physiology may be subject to question in the case of hemoglobin iron. More foods need to be studied in both species so that correlations can be made.

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APPENDIX A

Low iron diet

Ingredients	Percentage
Casein[x]	20
Corn oil (in glass container)	10
"Iron-free" mineral mixture[x]	2
Vitamin mixture[x]	1
Sucrose (analytical grade) or dextrose	67

[x] Obtained from Nutritional Biochemicals Corporation, Cleveland, Ohio, U.S.A.

APPENDIX B

Composition of scintillation solution

PPO[x]	4.00 g
POPOP[xx]	0.05 g
Toluene	1.00 liter

[x] PPO-2,5-Diphenyloxazole (Packard Instrument Company, Inc., Warrenville RD., Downers Grove, Illinois, U.S.A.).

[xx] Dimethyl-POPOP-1,4-bis- 2-(4-Methyl-5-Phenyloxazolyl) - Benzene (Packard Instrument Company, Inc., Warrenville RD., Downers Grove, Illinois, U.S.A.).

APPENDIX C

Optimal counter settings on Packard Tri-Carb Scintillation Spectrometer for the simultaneous determination of Fe^{55} and Fe^{59} radioactivity in the samples.

Fe^{55} channel (red)	
Window A width	100
Window B width	1000
Photomultiplier gain	80.0 %
Fe^{59} channel (green)	
Window C width	50
Window D width	550
Photomultiplier gain	2.2 %