PHYSIOLOGICAL AVAILABILITY OF IRON IN SOME MIDDLE EASTERN FOODSTUFFS

by

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IRON AVAILABILITY

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ABSTRACT

It has been shown that iron in certain foodstuffs is not utilized by experimental animals as readily as is iron in soluble inorganic iron salts. Also, a high incidence of iron-deficiency anemia has been reported in the Middle East even though dietary intake of iron appears to be adequate. This work was performed to establish a technique for the assessment of utilizable iron in foods commonly consumed in the area, and to study some of the dietary factors which are thought to interfere with iron utilization. Several methods devised by previous workers were examined; it was decided to employ the hemoglobin regeneration technique, using anemic rats for test animals.

A low-iron diet was formulated which would induce severe anemia in young rats in 3-5 weeks after weaning. In three consecutive experiments, seven foodstuffs were tested; these were: broad bean, chick pea, kidney bean, peeled lentil, okra, rice and wheat. The test diets were prepared so that each foodstuff provided all of iron at a level of 20 ppm. As an index of availability, gain in hemoglobin concentration per mg iron intake and gain in total hemoglobin per mg iron were determined. Comparisons were made with those values obtained from a control group

receiving the same level of iron in the form of ferrous sulfate.

The foodstuffs tested could be classified into three groups: 1) lentil, broad bean and wheat, 2) okra and rice, and 3) chick pea and kidney bean. The iron of first group was the most utilizable; that of the second group was poorly available to the rats; the third group showed intermediate availability. Assuming a value of 100 for ferrous sulfate, the per cent availability values were 75.6, 58.8, 51.0, 1.4, 78.2, 53.5 and 86.8 for broad bean, chick pea, kidney bean, okra, peeled lentil, rice plus ferrous sulfate and wheat when availability was expressed in terms of gain in hemoglobin concentration per mg iron. The corresponding values for availability in terms of gain in total hemoglobin per mg iron were 100.6, 76.2, 84.4, 2.8, 101.9, 59.7 and 80.1 respectively.

Since no linear relationship was observed between iron availability and food phytate-to-iron ratio, an experiment was designed to see the effect of added phytate on the utilization of ferrous sulfate. The results confirmed the previous observations; high levels of phytate had no effect on hemoglobin regeneration. No linear relationship was observed between physiological availability of iron to the rat and Ca:P, Ca:Fe or P:Fe ratios in the foodstuffs tested.

Availability values were not affected by sex or litter.

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INTRODUCTION

The role of iron in biological processes is most versatile. It performs its functions by virtue of its ability to take part in electron transfer systems, and to serve as a concentration of positive charges in solution, thus weakening covalent bonds, and bringing two molecules in close contact for interaction (1). Also, iron exhibits two electronic configurations, which vary in the number of unpaired electrons; therefore, it can form complexes that have different degrees of stability (1).

In hemoproteins iron participates in carrying oxygen, as in hemoglobin, in electron transfer as in the cytochromes, and as a prosthetic group in such enzymes as catalase and peroxidase. In non-heme compounds iron functions in photophosphorylation (2), and in nitrogen and sulfur fixation (3, 4), both as a part of ferridoxin. This metal also participates in such enzymatic systems as aconitase and phenolytic oxygenases (1).

A deficiency of dietary iron results in the impairment of many vital processes in the body. Hahn and Whipple (5) believe that, in iron deficiency, the concentration of iron-containing compounds in cells other than erythrocytes is preserved at the expense of iron in

hemoglobin. In contrast, Beutler (6) and Beutler and Blaisdell (7) showed that the hemoglobin synthesis mechanism is more successful in competition for iron; as a consequence, it may be true that deficiency symptoms are due to disturbances in the metabolism of the tissues rather than to anemia *per se*.

Symptoms of iron-deficiency anemia are the same as those observed in other nutritional anemias; that is, headache, palpitation, pallor and a sense of being "dead-tired" (8, 9). The anemia is microcytic, hypochromic in nature and is associated with an increased serum iron-binding capacity (9 - 13). Plasma iron is reduced (13). In the bone-marrow there is a condition of normoblastic hyperplasia, with little or no hemosiderin (12, 14). The sideroblasts may be virtually absent (14).

In children, anemia is manifested most frequently between 6 to 24 months of age during which the body iron stores have been depleted and the usual milk diet does not provide enough iron to meet the needs for growth (12). In men, anemia may occur at any age, but Brumfitt (10) says that men between 18 to 20 years of age are completing a period of adolescent growth during which the iron requirements are increased. There is an extra requirement for women due to menstruation and pregnancy. According to Underwood (12), there is a yearly menstrual loss of

200-400 mg and a loss of at least 350-450 mg for pregnancy; in the latter condition, the loss may be as high as 900 mg (15). Furthermore, in cases of chronic hemorrhages and parasitic infections, there is a greater demand for dietary iron.

Iron-deficiency anemia is a common deficiency disease throughout the world (16); it is a serious problem in North Africa and Asia (8). A high incidence of this type of anemia has been reported in several countries of the Middle East including Lebanon (17 - 19). In fact a recent study at the American University Hospital showed that, out of a group of 339 patients considered, 48% were diagnosed as suffering from iron-deficiency anemia (20). This high incidence is surprising in view of the fact that the soil, water and foods of the area are high in iron (21, 22). In addition, available dietary survey data indicate that iron intake is sufficient (17 - 19, 24, 25). Therefore, work is needed to study dietary factors which may be interfering with the physiological availability of food iron.

The present work was initiated with two objectives in mind: 1) to develop an experimental technique for the assessment of the physiological availability of food iron; and 2) to apply this technique to the study of iron availability in some common foodstuffs in relation to

inherent dietary factors which may affect the availability.

LITERATURE REVIEW

Iron Availability

The earliest attempts to investigate the availability of iron in various forms may be traced back to 1926 when Mitchell and Schmidt (26) found that ferric chloride and ferric ammonium citrate were better sources of iron than ferric oxide and ferric carbonate. In their bioassay method, these workers employed anemic rats as test animals and followed hemoglobin regeneration. Elvehjem and Sherman (27) and Sherman *et al.* (28) showed that organic forms of iron and insoluble inorganic iron salts were less efficient for restoring normal hemoglobin levels in anemic rats than were soluble inorganic iron compounds. Also, Underwood (29), working with anemic rats, found that ferrous and ferric iron were equally effective for normal growth, hemoglobin regeneration, and storage of iron in the liver.

In work with rats, Street (30) found that sodium iron pyrophosphate fed as such or in enriched bread was only 50% as available as ferrous or ferric sulfate. Fuhr and Steenbock (31) found that ferric phytate was 81% as available as ferrous ammonium sulfate, whereas Nakamura and Mitchell (32) reported 56% availability. These latter workers used ferric chloride as the standard and considered

only one week as the experimental period for the purposes of comparison. Also, in contrast to the results of Street, they showed that sodium iron pyrophosphate was as well utilized as ferric chloride. Freeman and Burrill (33), using as criteria both iron retention and hemoglobin regeneration in anemic rats, found that ferric chloride was more available than sodium ferric orthophosphate or ferric phosphate. In addition, they found that sodium iron pyrophosphate was the least available of the four forms.

Determination of the physiological availability of iron in foodstuffs was first attempted by Rose and Vahlteich (34). These workers fed whole wheat and oatmeal to anemic rats, as supplements to a milk diet at levels to provide 0.048, 0.096 and 0.192 mg of iron per rat per day. The rate of hemoglobin regeneration was found to be in proportion to the amount of the food consumed. After 6 weeks on diets providing 6 gm of either the wheat or oatmeal per day (0.192 mg iron), hemoglobin levels returned to normal. In similar experiments, Rose and Kung (35) obtained normal hemoglobin restoration by feeding 0.2 mg of wheat iron per rat per day for 6 weeks.

Elvehjem and co-workers (36) fed 0.15 mg of wheat iron per day and compared the results with those obtained from rats receiving 0.5 mg of iron as ferric chloride. From their results, they concluded that wheat was inferior to the

inorganic iron for hemoglobin regeneration. However, Rose et al. (37) showed that wheat, fed for 6 weeks, at a level to provide 0.19 mg iron daily was as effective in promoting hemoglobin regeneration in rats as was 0.20 mg per day of ferric chloride iron. These results were confirmed by Free and Bing (38), who employed a level of 0.25 mg of iron per day for 4 weeks, and by Smith and Otis (39). Furthermore, these latter workers found that barley iron was only 59% as available as wheat iron or ferric chloride.

According to Thompson and Raven (40), Sen found that wheat iron was only 53.6% available to anemic rats; similar results were obtained by Nakamura and Mitchell (32). Also, Sen reported corresponding values of 32.4% for rice, 40.0% for ferric phytate and 95.6% for egg yolk (40).

Raven and Thompson (41) working with certain grasses, clover and herb species, concluded that the iron in the more common species seemed to be only about 50% as available as the iron in ferric chloride.

According to Moore (15), some workers, using balance techniques on human subjects, found that only 13% of iron in spinach and 21% of that in beef was retained. Chodos et al. (42) fed to human subjects tracer doses of radioiron as inorganic salts or incorporated into food, and found that the mean percentage of the dose incorporated

into circulating hemoglobin was 0.85 for ferric chloride in one trial and 31.4 in another. Values of 8.7, 12.6, 1.4, 1.2 and 0.85 were obtained for ferrous chloride, egg plus ferrous chloride, egg, chard, and beet greens respectively. The corresponding values were higher when the same materials were tested on patients suffering from iron-deficiency anemia.

Moore (15) has collected figures from literature and from his own work for the percentage of radioiron utilized from foodstuffs by human subjects. The following values were cited: less than 4% from egg, lettuce, spinach, chard, beet greens, milk, and brewer's yeast; less than 10% from liver and enriched bread; and about 11% from muscle and hemoglobin. The corresponding values obtained with iron-deficient adults were much higher. In trials with normal children, the values reported were 5 to 10% for milk, egg plus 2 oz of milk, 2 oz of orange juice plus 1 piece of toast, egg plus 6 oz of milk, and chicken liver plus egg; 10 to 12% for egg, mixed cereals, oatmeal and rice plus Fe-59. As observed with adults, the corresponding values for anemic children were higher.

Methodology

Several methods have been devised for the assessment of iron availability. Hill (43) proposed the use of 2,2'-

dipyridyl reagent which he claimed would react with that form of food iron liberated in the reduced form in the stomach. With his method, Hill obtained results comparable with those from bioassay tests. Sherman et al. (28), Elvehjem and co-workers (36), Smith and Otis (39), Harris et al. (44), and Widdowson and McCance (45) confirmed Hill's observations. However, Hahn and Whipple (46) and Miller and Louis (47) found poor agreement between the chemical method and the bioassay technique. With respect to Hill's method, Thompson and Raven (40) say that availability values obtained chemically do not represent necessarily the iron which is utilizable under physiological conditions.

Of the several bioassay techniques, balance studies with normal animals give almost nil availability values because of very low iron absorption (40).

Some workers (49, 50) have used the technique of total iron retention; that is, sacrificing the animal and determining total iron in the carcass. This technique has the disadvantage of ignoring intestinal iron secretions which, at some time previous to sacrificing, had been absorbed (40).

A method employed by many workers (27 - 29, 36, 40, 44) is one in which young rats are made anemic by feeding an all-milk diet, and then are put on diets containing

iron from different sources. In this method, the rate of hemoglobin regeneration is determined and compared with that of a group receiving an inorganic soluble salt of iron. Several merits have been attributed to the latter technique. First, a young anemic rat requires iron for growth and recovery, and it makes the best use of the iron ingested. In an animal under these circumstances, erythropoeitic activity is stimulated and, as a result, there is efficient iron absorption (52, 53). Also, the method is more convenient than techniques involving balance and slaughter, and it permits several measurements on the same animal.

In the hemoglobin regeneration technique, various levels of iron have been adopted for a normal anemia recovery process. These levels vary from as little as 0.08 mg (47) to 0.50 mg per rat per day (40); most workers have recommended 0.25 to 0.30 mg (28, 37, 39, 54). In several studies, a level of 0.2 mg has been found to support normal hemoglobin regeneration when the iron source was a soluble salt (35, 37, 40).

The most recent type of bioassay method for iron availability involves the use of radioiron incorporated into foodstuffs and fed to normal or anemic subjects (42, 55, 56). The double-isotope test (56), in which one isotope is incorporated into a foodstuff and the other is

injected, could be used as a device for the measurement of iron absorption. In balance studies using a single isotope, the percentage of the dose fed is found either by considering the recovery in the feces or in the circulating hemoglobin (52).

Dietary Factors Affecting Iron Availability

Many attempts have been made to investigate dietary factors which may impair iron utilization. In one study, high levels of calcium were found to inhibit iron absorption in rats (57); these workers claimed that the calcium competed with iron for absorption. Kletzein (50) showed that the feeding of 1 and 3% calcium carbonate to rats gave lower tissue iron values; the same results were observed with calcium lactate and with calcium chloride and triphosphate fed at levels equivalent to 2½ and 3% of calcium carbonate respectively. Calcium phosphate did not give the same results. In contrast, Gubler (58), Foy and Kandi (59), and Apte and Venkatachalam (60) state that high dietary calcium may enhance iron absorption in man. According to some authors (50, 59, 60), calcium in excess might be expected to form calcium phytate and phosphate leaving less of these compounds to combine with iron. The iron would therefore be free for absorption.

Considering the effect of phosphorus alone on iron

absorption, again there is a great deal of controversy. Chapman and Campbell (57) found that high levels of dietary phosphates did not inhibit iron availability in rats. Also, Haas (62) reported that condensed phosphates, either in food or as supplements did not affect iron absorption in anemic rats. However, Hegsted et al. (63) reported inhibitory effects of phosphate on iron absorption.

Tompsett (49) found that phosphatides and phosphoproteins in egg had an inhibitory effect on iron utilization in mice.

The effect of phytic acid on iron absorption has been the topic of a great deal of investigation. Fuhr and Steenbock (31) showed that, with optimal levels of dietary calcium, addition of phytic acid did not alter the amount of total body iron in rats. However, there appeared to be a reduction in the amount of hemoglobin produced. Walker et al. (64) reported that retention of iron in man was virtually the same with high and low phytate-phosphorus diets.

In studies with radioiron, Sharpe et al. (65) found no correlation between the phytate content of rolled oats and iron availability in human subjects. However, in diets to which they added sodium phytate, they found that the soluble phytate interfered with iron absorption. Working

with rice diets, Sathe and Krishnamurthy (66) reported that, in rats, the higher the level of phytin phosphorus, the less the absorption of iron. Hussain and Patwardhan (67) demonstrated that, when 8% of the phosphorus in the diet was in the form of phytin, the average retention of iron by human subjects was 2.483 mg ; when 40% of phosphorus was from phytin, only 0.173 mg was retained. However, Foy and co-workers (68) were unable to obtain consistent effects of phytate on absorption of radioiron in human subjects. Turnbull et al. (69) found that phytate did not interfere with the utilization of iron from hemoglobin.

MATERIALS AND METHODS

Experimental Animals

Albino rats of the Sprague-Dawley strain[1] were used throughout; unless otherwise specified, the animals were bred within the departmental colony. The rats were housed in an air-conditioned room in which the temperature was held at 21± 1°C; relative humidity was about 60%.

For breeding, one adult male was placed in a cage with 3 or 4 females for one week. The males were circulated among the breeding cages during this period to assure successful mating. A few days prior to parturition, each pregnant rat was placed in an individual cage containing nesting material. Ten days after parturition, each mother, along with her litter, was transferred to a stainless steel cage in which deionized water and a low iron diet were provided *ad libitum*. Every day each mother was isolated and fed on stock diet[2] for about 2 hours to assure adequate iron intake. At the age of 21-23 days, the young rats were weaned onto the anemia producing diet; normally a period of 3-5 weeks on this diet was required to develop severe anemia.

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1. Obtained from Animal Suppliers (London) Ltd.
2. Obtained from Vitasni Feed Company, Beirut.

After hemoglobin levels fell to a satisfactory level (4-7 gm%), diets were assigned, depending on the experiment, according to either a randomized block design on the basis of hemoglobin, or to a split-plot design on the basis of sex and hemoglobin, or sex, hemoglobin, and littermate. The animals were individually housed in iron-free cages and received test diets and demineralized water ad libitum. Feed consumption, spillage, and weight gains were determined weekly.

Unless otherwise specified, hemoglobin determinations were made on each animal, at weekly intervals for 4-5 weeks.

Cages

The cages used throughout were made of stainless steel with mesh-bottoms of chrome-plated brass[3].

Diets

The basal low iron diet developed was as follows:

Ingredients	Percentage
Casein[4]	20
Corn oil (in glass container)	10
"Iron-free" salt mixture[4]	2
Vitamin mixture[4]	1
Sucrose (analytical grade)	67

3. Fabricated in the School of Engineering, American University of Beirut.
4. Obtained from Nutritional Biochemicals Corporation, Cleveland, Ohio, U.S.A.

Analyses made on this diet at various times showed that the iron content ranged from 2-6 ppm, depending on the source of casein. For the preparation of the test diets, the foodstuff in question was added at the expense of sucrose to provide 20 ppm of iron. The control diet was prepared by adding to the basal diet 20 ppm of iron in the form of ferrous sulfate solution. The solution was pre-mixed with the casein which was then mixed thoroughly with the remainder of the diet.

For phytate experiments, salt mixture was prepared according to USP XIV except that iron and phosphorus were deleted; iron and phosphorus (either as phosphate or phytate) were added at the required levels according to the experimental design.

For mixing diets, a Hobart food mixer equipped with a stainless steel mixing bowl was employed. Diets were stored in plastic containers previously rinsed with deionized water.

Materials

The foodstuffs studied were: broad bean, chick pea, kidney bean, okra, peeled lentil, rice, and wheat; the samples were purchased from the Beirut retail market. The materials were first washed with distilled water and cooked in a minimum amount of demineralized water in glass

containers. After cooking, they were dried in a force draft oven at 90°C and ground to a fine mesh in a stainless steel grinding mill. All ground samples were analyzed for iron, phosphorus, calcium and phytate.

Collection of Blood

For blood collection, the animal was held immobile in a specially designed plastic box. The tail was immersed in warm water (45°C) for 1-2 minutes after which it was dried and a small portion of the tip amputated with surgical scissors. A few drops of blood were collected in a heparinized tube; care was taken to avoid excessive bleeding.

Analytical Methods

Hemoglobin was measured by the cyanomethemoglobin method (72). By means of a Sahli hemoglobin pipet 0.02 ml of blood was transferred into a previously matched colorimeter tube containing 5 ml of a solution of the following composition (per liter): $NaHCO_3$, 1 gm; KCN, 50 mg; and $K_3Fe(CN)_6$, 200 mg. The blood was mixed thoroughly with the contents of the tube; optical density was measured after 30 minutes at 540 mμ in a Unicam spectrophotometer, Model SP600.

Iron and phosphorus were determined according to

A.O.A.C.[5] methods (13). Food samples were digested in a mixture of concentrated nitric and perchloric acid to oxidize all the organic matter and release iron in an inorganic form. The iron was reduced to the ferrous form with hydroxylamine hydrochloride; o-phenanthroline was added and a red color was produced after addition of ammonium acetate. After 30 minutes, optical density was measured at 530mμ.

Phosphorus was measured in the digestion mixture by adding molybdic acid to produce phosphomolybdate which was then reduced with stannous chloride. The intensity of the blue color developed was measured within 30 minutes at 660 mμ.

Phytate was determined by the method of Casares and Moreneo (74). The sample of ground foodstuff was extracted with 2% hydrochloric acid and filtered. The filtrate was then diluted with water, warmed, and titrated with a solution of ferric chloride using sodium salicylate as an indicator.

For calcium analysis the method of the California Agricultural Experiment Station (75) was used. The plant material was dry-ashed and the calcium precipitated as the oxalate, calcium was then determined by titrating with a

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5. Association of Official Agricultural Chemists.

solution of standard potassium permanganate.

All glassware used was cleaned with cleaning solution and rinsed repeatedly with deionized water.

RESULTS

The results of the present work have been reduced to tabular and graphic forms and are presented on the following pages in Tables 1 - 9 and Figures 1 - 6. The discussion of these results is presented in the next section of the thesis.

Table 1. Iron content of some feed ingredients.

Ingredient	Iron Content
	mg/100 gm
Corn starch, local	3.82
Corn oil, "Mazola" (in metal container)	1.55
Low-iron mineral mixture, Nutritional Biochemical Co.	10.06
Casein, General Biochemicals Inc.	12.23
Casein, Sheffield	2.20
Dextrose, monohydrate, Hopkins & Williams	2.25
Dried whole milk "Klim"	3.50
Dried skim milk, local	2.00
Sucrose, granulated, "Jack Frost"	0.77
Sucrose, powdered, "Domino"	1.10
Sucrose, local	2.00

Table 2. Calcium, iron, phosphorus, and phytate content of the various foodstuffs tested[1].

Foodstuff	Calcium	Iron	Phosphorus	Phytate
Broad bean	144	6.08	567	1348
Chick pea	136	6.39	375	738
Kidney bean	182	5.74	472	984
Okra	1845	13.63	662	2216
Peeled lentil	58	8.72	418	549
Rice	40	1.26	206	127
Wheat	234	3.32	326	728

1. Expressed in mg per 100 gm dry weight.

Table 3. Average weight and total feed intake of rats in three experiments.

Experiment	Diet	Number of animals	Initial	Weeks 1	2	3	4	Total feed intake
			Weight in grams					grams
I	Basal+$FeSO_4$ [1]	6	33	54	83	115	146	309
	Broad bean	8	34	66	110	145	167	318
	Kidney bean	8	35	64	102	139	167	294
	Peeled lentil	8	36	66	104	136	161	294
II	Basal+$FeSO_4$ [1]	8	66	104	141	178	209	440
	Chick pea	8	68	115	148	185	208	408
III	Basal+$FeSO_4$ [1]	7	89	121	155	201	212	380
	Okra	8	86	114	146	177	192	348
	Rice+$FeSO_4$ [2]	8	84	116	152	178	183	348
	Wheat	8	87	132	160	196	192	394

1. $FeSO_4$ provided 20 ppm of iron.
2. $FeSO_4$ provided 12 ppm of iron.

Table 4. Average iron intake and gain in hemoglobin concentration and total hemoglobin per mg iron intake in Experiments I, II, and III.

Experiment	Diet[1]	Number of animals	Iron level in diet[2]	Iron intake	Gain in hemoglobin concentration	Gain in total hemoglobin
			ppm	mg	gm%/mg Fe	gm/mg Fe
I	Basal+$FeSO_4$	6	26.54	8.17	0.772	0.154
	Broad bean	8	26.83	8.37	0.584[3]	0.155
	Kidney bean	8	28.43	8.36	0.394[4]	0.130[3]
	Peeled lentil	8	26.69	7.93	0.604[3]	0.157
II	Basal+$FeSO_4$	8	22.75	10.01	0.729	0.164
	Chick pea	8	21.83	8.91	0.429[4]	0.125[3]
III	Basal+$FeSO_4$	7	22.82	8.67	0.908	0.186
	Okra	8	23.76	8.27	0.013[4]	0.052[4]
	Rice+$FeSO_4$	8	21.87	7.61	0.486[4]	0.111[4]
	Wheat	8	22.81	8.99	0.788	0.149[3]

1. All diets had 20 ppm of inorganic or food iron.
2. Determined values.
3. Significantly different from Basal diet+$FeSO_4$ at 5% level.
4. Significantly different from Basal diet+$FeSO_4$ at 1% level.

Table 5. Analysis of variance for Experiment I

Factor	Gain in hemoglobin concentration per mg iron intake[1]				Gain in total hemoglobin per mg iron intake[2]			
	Sum of squares	d.f.	Variance	F	Sum of squares	d.f.	Variance	F
Replication	0.151	3	0.050	2.08	0.000986	3	0.000329	1.75
Sex	0.040	1	0.040	1.67	0.000734	1	0.000734	3.90
Error (a)	0.071	3	0.024	-	0.000564	3	0.000188	-
Diet	0.555	3	0.185	7.71^{xx}	0.003564	3	0.001188	3.49^{x}
Sex X Diet	0.054	3	0.018	0.08	0.000726	3	0.000242	0.71
Error (b)	0.376	16[3]	0.024	-	0.005443	16[3]	0.000340	-
Total	1.247	31			0.012017	31		

^{x} Significant at the 5% level.

^{xx} Significant at the 1% level.

1. L.S.D. at the 5% level 0.164; at 1% 0.226.

2. L.S.D. at the 5% level 0.020.

3. Two less because of the estimation of the two missing data.

Table 6. Analysis of variance for Experiment III

Factor	Gain in hemoglobin concentration per mg iron intake[1]				Gain in total hemoglobin per mg iron intake[2]			
	Sum of squares	d.f.	Variance	F	Sum of squares	d.f.	Variance	F
Litter	0.128	3	0.043	1.13	0.003631	3	0.001210	3.88
Sex	0.095	1	0.095	2.50	0.000023	1	0.000023	0.74
Error (a)	0.113	3	0.038	-	0.000937	3	0.000312	-
Diet	3.758	3	1.253	56.95^{xx}	0.077487	3	0.025829	30.32^{xx}
Sex X Diet	0.013	3	0.004	0.18	0.002471	3	0.000824	0.97
Error (b)	0.380	17[3]	0.022		0.014490	17[3]	0.000852	-
Total	4.487	31			0.099039	31		

^{xx} Significant at the 1% level.

1. L.S.D. at the 5% level 0.156; at the 1% 0.215.

2. L.S.D. at the 5% level 0.031; at the 1% 0.042.

3. One less because of the estimation of missing datum.

Table 7. Physiological availability of iron in some foodstuffs consumed commonly in the Middle East.

Foodstuff	Per Cent Availability[1]	
	Gain in hemoglobin concentration per mg Fe	Gain in total hemoglobin per mg Fe
Broad bean	75.6	100.6
Chick pea	58.8	76.2
Kidney bean	51.0	84.4
Okra	1.4	2.8
Peeled lentil	78.2	101.9
Rice+$FeSO_4$[2]	53.5	59.7
Wheat	86.8	80.1

1. Assuming a value of 100% availability for ferrous sulfate.
2. Ferrous sulfate provided 12 ppm of iron.

Table 8. Growth and feed intake of animals fed the basal diet containing various levels of phytate.

Diet[1,2]	Dietary phytate[3]	Number of animals	Initial	Weeks 1	2	3	4	5	Total feed intake
	mg/100 gm		Weight in grams						grams
1- Basal	-	7	123	145	182	227	257	284	519
2- Basal+15% P as phytate	230	7	129	152	188	228	257	273	521
3- Basal+45% P as phytate	645	6	118	142	184	214	253	261	511
4- Basal+45% P as phytate[4]	720	8	78	112	135	157	166	-	294

1. All diets contained 20 ppm Fe as $FeSO_4$.
2. Phytate was added at the desired level at the expense of inorganic phosphate.
3. Determined values.
4. Treatment 3 repeated.

Table 9. Relationship of various chemical factors to the physiological availability of iron in various foodstuffs.

Foodstuff	Per Cent Availability		P:Fe	Ca:P	Ca:Fe
	Gain in hemoglobin concentration per mg iron intake	Gain in total hemoglobin per mg iron intake			
Rice	0.0	0.0	161.5	0.19	31.7
Okra	1.4	2.8	48.6	2.79	135.4
Kidney bean	51.0	84.4	82.3	0.39	31.7
Chick pea	58.8	76.2	58.5	0.36	21.3
Broad bean	75.6	100.6	93.2	0.26	23.7
Peeled lentil	78.2	101.9	47.9	0.14	6.6
Wheat	86.8	80.1	98.3	0.72	70.5

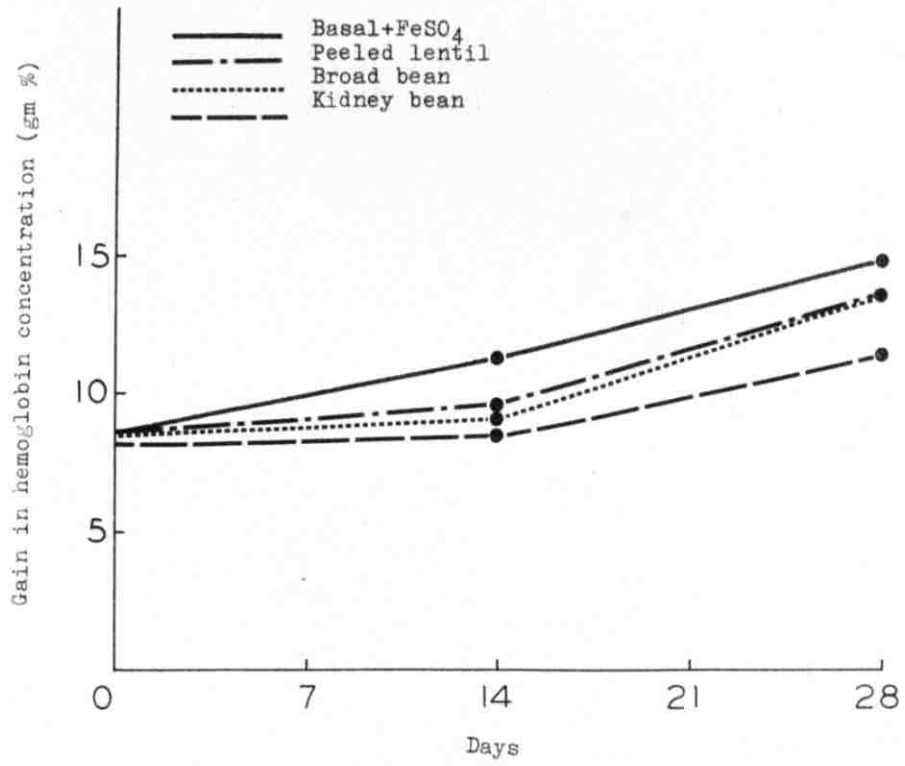

Figure 1. Hemoglobin regeneration in anemic rats fed diets containing 20 ppm of iron.

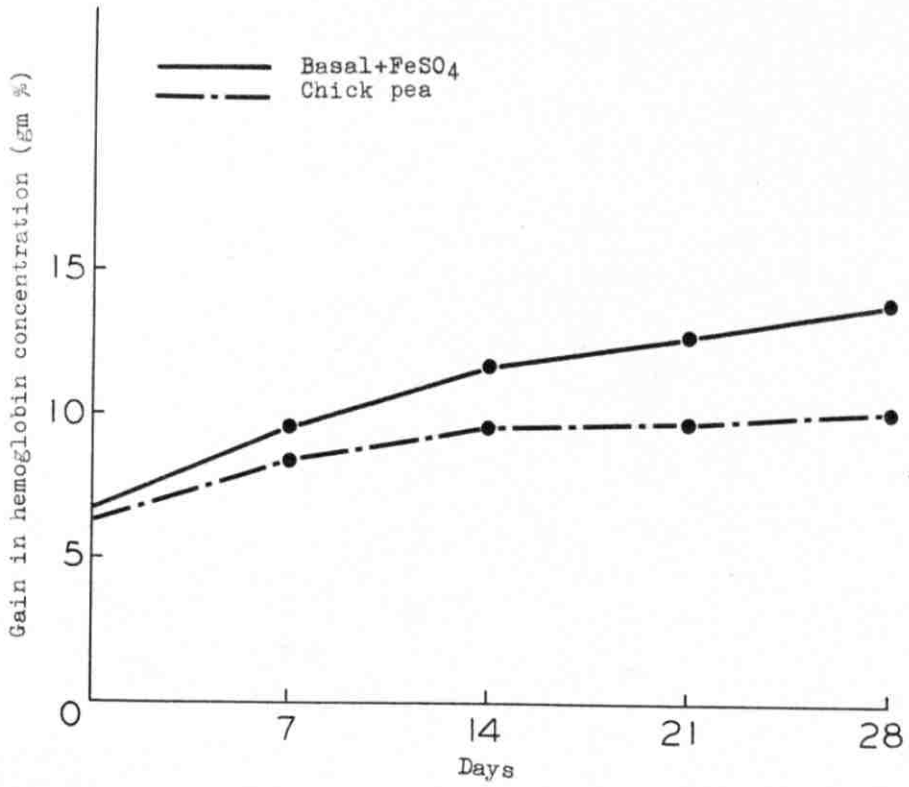

Figure 2. Hemoglobin regeneration in anemic rats fed diets containing 20 ppm of iron.

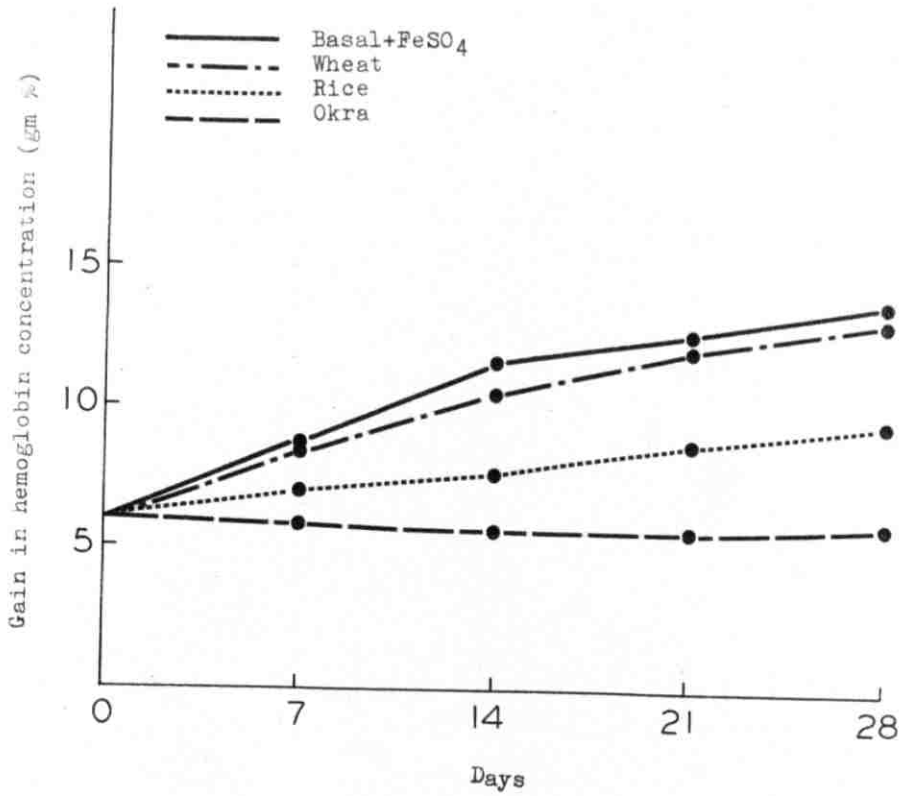

Figure 3. Hemoglobin regeneration in anemic rats fed diets containing 20 ppm of iron.

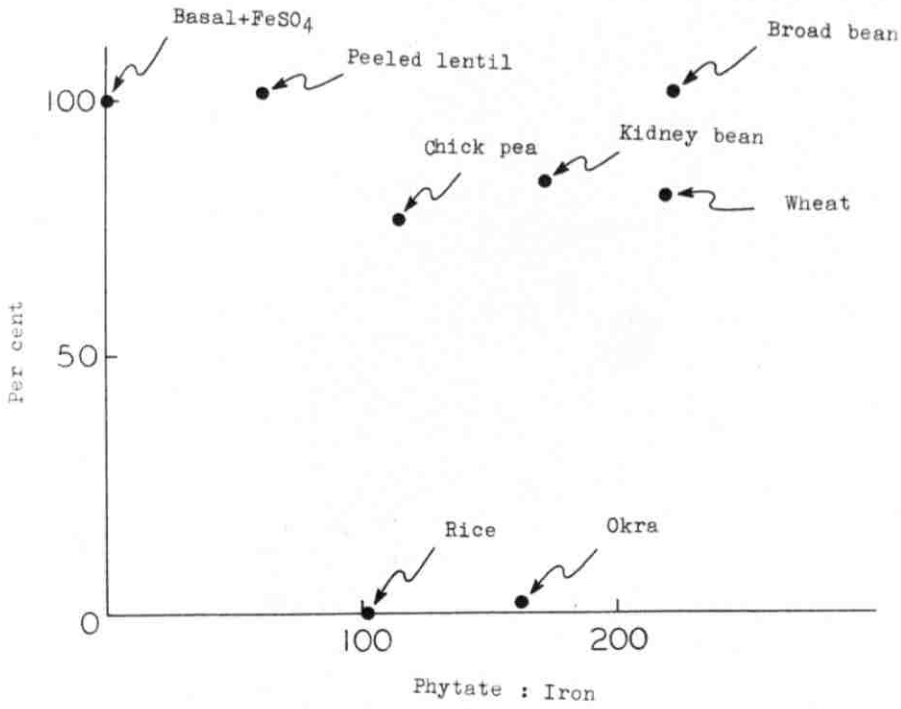

Figure 4. Relationship of phytate-to-iron ratio and physiological availability of iron expressed as gain in total hemoglobin per mg iron intake.

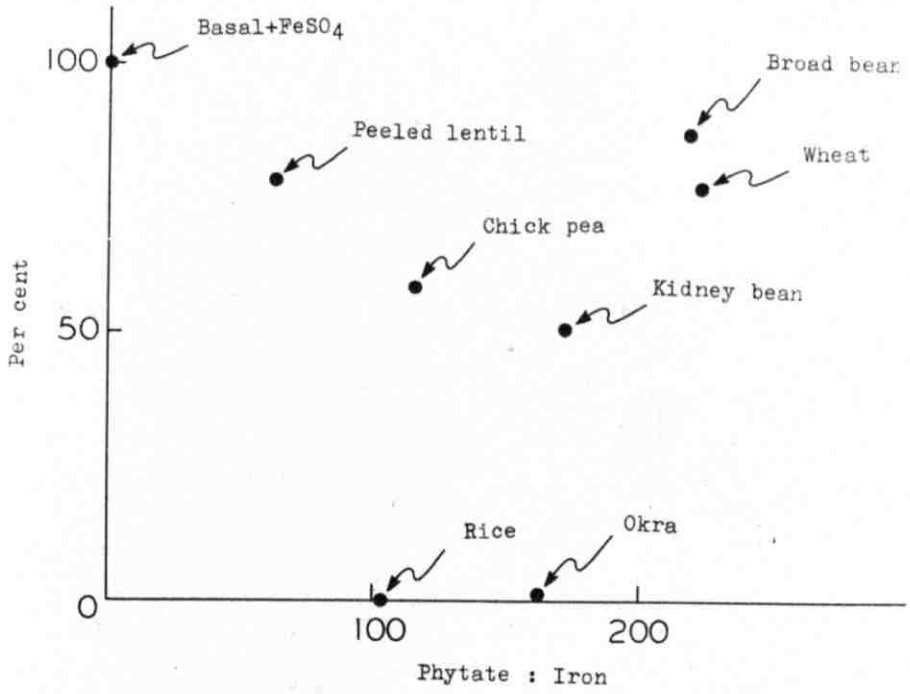

Figure 5. Relationship of phytate-to-iron ratio and physiological availability of iron expressed as gain in hemoglobin concentration per mg iron intake.

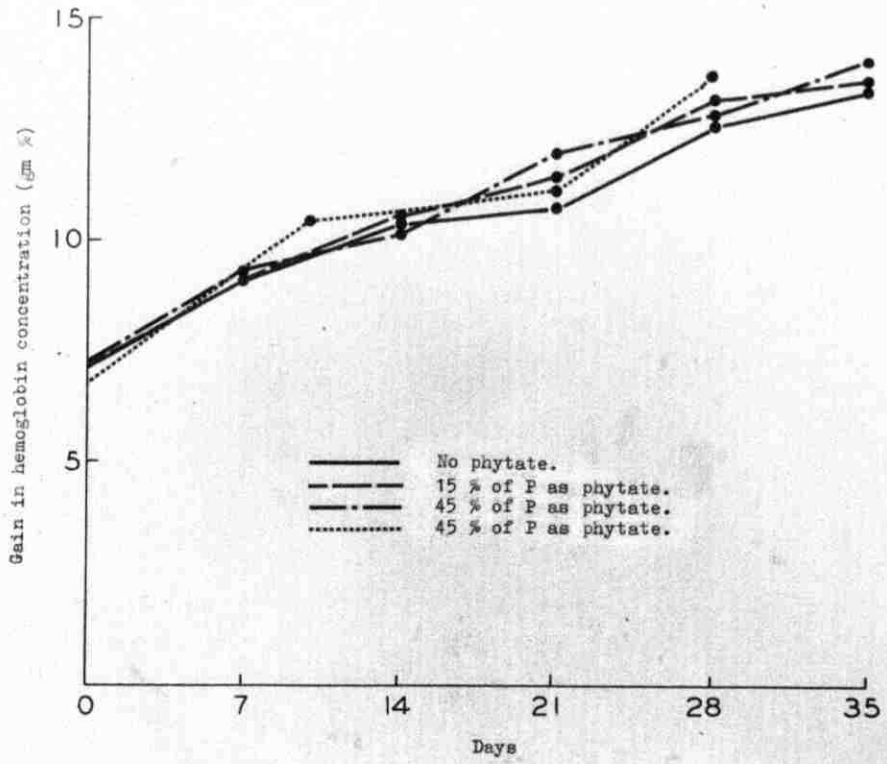

Figure 6. Effect of sodium phytate on hemoglobin regeneration rate of anemic rats fed diets containing 20 ppm of iron as $FeSO_4$.

DISCUSSION

Development of Low-Iron Diet

Milk, in one form or other, with or without mineral supplement, has been employed widely as the classical diet for inducing iron-deficiency anemia (36-38, 76-79). However, several trials using milk failed under the present experimental conditions. The animals did not grow properly and, in many instances, many developed diarrhea and died. Furthermore, a fresh supply of liquid milk uncontaminated with iron was not available. Commercial brands of skim milk or dried whole milk contained too much iron to be of any use. Consequently, it was necessary to develop a solid diet which would have the following characteristics: 1) it would be sufficiently low in iron to induce anemia, and 2) it would support normal growth.

Several feed ingredients were analyzed for iron content (Table 1). However, because of iron contamination, most of them had to be rejected, before a diet was formulated according to the composition given under Materials and Methods. This diet proved to fulfill the above criteria for both anemia production and growth.

Effect of Food Iron on Hemoglobin Regeneration

Three experiments were undertaken in which 7 foodstuffs commonly consumed in the Middle East were tested. These experiments represented the pattern in which the technique, the establishment of which was the main purpose of this work, was examined.

Analyses were made on the foodstuffs to be tested for calcium, iron, phosphorus and phytate content; the results are shown in Table 2. Rice had the lowest level of all the items. Okra was the richest one in iron, calcium, phosphorus and phytate. Since one of the chief interests in the work was the effect of phytate, the foodstuffs were chosen so that there was a wide variation in phytate content.

Experiment I

This experiment represented the most preliminary part of the work on the physiological availability of food iron. Since previous trials involving the weaning of rats onto whole cow's milk had failed, these animals received cow's milk, as a supplement to mother's milk, only until they were weaned. At that time, they were placed on experiment; animals for subsequent experiments were kept on the low-iron solid diet for several weeks after weaning to induce more severe anemia.

The foodstuffs tested in Experiment I were broad bean (Vicia faba), kidney bean (Phaseolus vulgaris) and peeled lentil (Lens esculenta). The diets were assigned at random according to hemoglobin and sex. Since the animals were not kept on a low iron diet after weaning, the initial hemoglobin levels were high relative to subsequent experiments. The values were 8.17, 8.44, 8.52 and 8.66 gm% for the groups on kidney bean, basal plus ferrous sulfate, broad bean and peeled lentil respectively. Each group consisted of 4 male and 4 female animals. During the experimental period, hemoglobin was determined twice: once after 14 days and the second time at the end of the experiment (28 days).

The curves in Figure I show the hemoglobin regeneration of the groups on the various diets. As seen from these curves, the hemoglobin regeneration rate of the group receiving iron from ferrous sulfate was the most rapid. The rate was slowest for the group on kidney bean. For the groups on broad bean and lentil, the regeneration patterns were similar. Final hemoglobin levels were 11.46, 13.41, 13.45 and 14.75 gm% for the groups fed kidney bean, broad bean, lentil and ferrous sulfate diets respectively.

In Table 3, data for the 3 experiments are shown for growth and total feed intake of the animals. In the first experiment, the rats on basal diet failed to grow as

fast as the others probably because the level of protein (18% casein) was not high enough for maximum growth. In subsequent experiments, the casein level was raised to 20%; the latter level proved to support optimum growth.

In order to compare the relative effectiveness of various foodstuffs in promoting hemoglobin regeneration, several workers fed a certain amount of iron per day from a given source, and used gain in hemoglobin concentration for comparison (34, 36, 38). Thompson and Raven (40) used both gain in hemoglobin concentration and gain in total hemoglobin per mg iron intake. In the present work, it was decided to use the criteria of the latter workers. For convenience in determining iron intake, rats were fed ad libitum and feed consumption was carefully measured. This procedure allowed the calculation of gain in hemoglobin concentration per mg iron intake; thus the effect of differences in feed intake on various diets was eliminated. To minimize the effect of different growth rates, gain in total hemoglobin per mg iron intake was calculated by using a value of 6.7 ml of blood per 100 gm body weight; this figure has been established by several workers (80 - 82).

Data for iron intake, gain in hemoglobin concentration and total hemoglobin per mg iron are shown in Table 4; the analysis of variance for the results is presented in Table 5. During the experiment, one female

rat died after one week in the control group; in the same group, one male developed diarrhea and consequently, it was excluded from the calculations. The two missing values were estimated according to the method described by Cochran and Cox (71).

Analysis of variance showed that there was a significant difference between the availability of the iron in both broad bean and peeled lentil and that of ferrous sulfate, at the 5% level, only for gain in hemoglobin concentration per mg iron. However, the difference between kidney bean and ferrous sulfate was significant at 1% for gain in hemoglobin concentration and at 5% for total hemoglobin per mg iron. The differences between the availability values for kidney bean and either broad bean or peeled lentil were significant at 5% for both indices of availability (Table 4).

Analysis of the data showed that there was no significant relationship between sex and utilization of iron from various sources (Table 5). Such a conclusion had been made previously by Ascham and co-workers (83) and Free and Bing (84). However, Rose and Kung (35) found that female rats had higher values than males when availability was expressed in terms of gm hemoglobin gained per gm body weight. Also, Smith and Otis (39) found that hemoglobin response of female rats was greater than that of males.

Rose and Hubbell (85) showed that female rats stored more iron than males, and Widdowson and McCance (86) observed that female rats exhibited a more efficient utilization of iron for hemoglobin regeneration than did males. Finally, Thompson and Raven (40), believing in the effect of sex on iron absorption, used only 4 male animals per group, a number which was later increased to 6.

In view of the results of this experiment and of others, and because of the controversy cited, it appeared justifiable to use relatively large groups of mixed sex. Besides, in a practical situation, the number of animals of the same sex may be limited. The method, as established here, therefore, involves the use of equal number of male and female rats in each group.

The results of this preliminary experiment along with those of other workers indicated the experimental conditions which seemed adequate for subsequent work in studying the physiological availability of iron. It was decided that: 1) rats were adequate as experimental animals; 2) groups of 8 animals (4 males and 4 females) would be sufficient; 3) animals should be weaned for some time onto a low-iron diet to produce severe anemia before going on experiment; 4) the experimental period should be 28 days; and 5) the level of 20 ppm of dietary iron, if readily available, could be used for satisfactory hemoglobin regeneration.

Experiment II

In this experiment the conditions were essentially the same as in Experiment I, except that the animals were kept on the low-iron diet after weaning to induce more severe anemia. This procedure proved to be successful and, in some animals, hemoglobin concentration dropped to as low as 3 gm% after 5 weeks.

Because of breeding problems, there were only 3 litter groups of rats available for this experiment. Therefore, only two groups were chosen one for the control and one for a test foodstuff (chick pea, Cicer arietinum). One litter provided 3 females and 1 male for each diet, whereas the other 2 litters each provided 1 male and 1 female for each of the two treatments. The diets were assigned randomly according to sex and hemoglobin within the same litter.

The average weight gain and total feed intake of the animals are shown in Table 3. Since the rats were kept on the low iron diet for several weeks after weaning, the initial body weights were higher than in Experiment I. Hemoglobin dilution, brought about by the rapid growth of the animals in the presence of low dietary iron, resulted in severe anemia. Initial mean hemoglobin values were 6.22 and 6.65 gm% for the groups on the chick pea and basal diet respectively.

In Figure 2 are shown the hemoglobin restoration patterns. The rate of hemoglobin regeneration in animals fed the chick pea diet was much slower than in those on the control diet. At the end of 4 weeks, the average value for the group receiving ferrous sulfate was 13.95 gm% and for chick pea, 10.04.

Data for iron intake, gain in hemoglobin concentration and total hemoglobin per mg iron are shown in Table 4. Student's "t" test showed that there was a highly significant difference between gain in hemoglobin concentration per mg iron in rats on the two diets. Also, the average values for total hemoglobin per mg iron were significantly different at 5%.

Experiment III

The plan of this experiment was essentially the same as that of Experiment II; four litters, each providing 4 males and 4 females were selected. After the animals were rendered anemic, treatments were assigned randomly according to hemoglobin, sex, and litter. The design was a split-plot in which litter represented replications, sex the main plot, and diet the sub-plot factors. The foodstuffs tested were okra,(*Hibiscus esculentus*), rice (*Oryza sativa*), and wheat (*Triticum vulgare*). Since the amount of rice incorporated into the

diet provided only 8 ppm of iron, ferrous sulfate was added to the rice diet to raise the level of total iron to 20 ppm . The average initial hemoglobin concentrations were 5.84, 5.86, 5.91 and 6.09 for the groups on rice, okra, ferrous sulfate, and wheat respectively.

The hemoglobin regeneration response to the diets are shown in Figure 3. The mean hemoglobin levels in all five determinations throughout the experiment were highest for the group which received iron from ferrous sulfate. The final hemoglobin concentrations were 5.97, 9.54, 13.17 and 13.80 gm% for the groups on okra, rice plus ferrous sulfate, wheat and basal plus ferrous sulfate respectively. Compared to okra and rice, wheat was the best source of utilizable food iron. The iron in okra failed to raise the hemoglobin concentration above the initial level. Despite the fact that 12 ppm of iron was added to the rice diet as ferrous sulfate, this diet was much inferior to the wheat diet in promoting hemoglobin regeneration.

The data for growth and average total feed intake are shown in Table 3. The mean weight for the group on rice diet was lowest and that of the group on basal was highest. The mean total feed intake was highest for the animals on the wheat diet.

Data for iron intake, gain in hemoglobin concentration and total hemoglobin per mg iron, are shown in

Table 4. The differences between the control values and the values for both okra and rice plus ferrous sulfate were significant at the 1% level for both indices of iron utilization. The difference between the availability of wheat iron and that of ferrous sulfate was not significant for gain in hemoglobin concentration per mg iron. However, a significant difference was observed for gain in total hemoglobin per mg iron. This latter observation apparently was due to the lower mean final body weight for the group on the wheat diet (Table 3).

If it can be assumed that iron from ferrous sulfate had priority in being utilized, the gain in hemoglobin concentration and total hemoglobin per mg iron was such that little or none of the iron in rice appeared to be available. Thompson and Raven (40) reported that Sen obtained 32.4% availability for iron in rice; however, the details of his work are not available. The observation that wheat iron compared favorably to ferrous sulfate agrees with results of Rose and Kung (35), Rose *et al.* (37). Free and Bing (38), and Smith and Otis (39). However, the present results are not in accord with those obtained by Elvehjem and co-workers (36). Also, other workers have reported that the iron from wheat was not as available as inorganic iron (32, 40).

Analysis of variance (Table 6) showed that, as

previously observed, there was no relationship between litter and dietary iron sources. Thompson and Raven (40) found such a relationship in their work although their results were not consistent.

The values for per cent availability of the 7 foodstuffs tested are presented in Table 7. The percentages were calculated using the assumed value of 100 for ferrous sulfate; each foodstuff was compared to the ferrous sulfate control of the experiment in which it was tested. When expressed as gain in hemoglobin concentration per mg iron, wheat had the highest value and okra the lowest. The same trend was not observed when the percentages were expressed on the basis of gain in total hemoglobin per mg iron. However, those foods which showed high availability with one index, (wheat, lentil and broad bean) had high values with the other. Okra had low values with both indices. It appeared that the iron in rice was not available, since relatively low values were obtained even when 12 ppm of iron was added to the diet.

Effect of Food Phytate on Iron Absorption

As seen in Figures 4 and 5, there was no linear relationship between the physiological availability of iron and the phytate-to-iron ratio in the various foodstuffs tested. This observation agrees with the results

of several workers. Walker *et al.* (64) reported that iron retention was virtually the same when diets with high and low phytate-phosphorus were fed to human subjects; Sharpe *et al.* (65) found no correlation between iron absorption in man and the phytate content of rolled oats. Also, Turnbull and co-workers (69) reported that absorption of iron from hemoglobin was not affected by phytate.

In contrast, Sathe and Krishnamurthy (66) observed that, in rats, the higher the level of food phytin, the less the absorption of iron. However, these latter workers used rice as the only source of phytate, and it could well have been that factors other than phytate were interfering with iron absorption. Furthermore, they used small numbers of animals and there was a great deal of overlapping in their results; there was no statistical treatment of the data. Hussain and Patwardhan (67) performed balance studies on normal human subjects, and reported that phytate had an adverse effect on iron utilization. Foy and co-workers (68) were unable to obtain consistent results when they tested the effect of phytate on absorption of radioiron in man. Apte and Venkatachalam (60), on the basis of data from balance studies on normal human subjects, recommended a high level of dietary iron because of the possible inhibitory effect of phytate.

A great deal of the controversy seems to arise

from differences in experimental design and interpretation of results. For example, Sahte and Krishnamurthy used rice phytate as the only variable. In this instance, other factors in rice which may have affected iron absorption were not controlled. Also, coincidence may be a factor in the controversy. For example, if, in the present work, one considered data from only lentil, chick pea and kidney bean, (Figure 5), it could be concluded that there was an inverse relationship between food phytate and iron utilization.

Since the present results, along with others discussed, were not conclusive, it seemed necessary to examine the relationship of phytate to iron availability under experimental conditions which would permit the control of all factors, so that the level of phytate would be the only variable. To do so, the following experiment was designed: Three groups of 7 anemic rats each were selected; the animals were obtained as weanlings from a commercial firm. Diets were assigned randomly according to hemoglobin. All the diets had 20 ppm of iron as ferrous sulfate, and 0.4% phosphorus as either inorganic phosphate alone or as phosphate plus phytate. Diet 1 had no phytate, diet 2 had 15% of the phosphorus as sodium phytate and in diet 3, 45% of the phosphorus was in the form of phytate. The experiment lasted for 35 days and hemoglobin was

determined weekly.

Growth and feed intake data are shown in Table 8; also shown in Table 8 are data on growth and feed intake of a single group of anemic rats which represented a later repetition of diet 3 (45% phosphorus as phytate). The animals on all diets gained normally. Since the animals of the fourth group were younger when they were put on experiment, the initial and final weights were lower than those of the other groups.

Hemoglobin regeneration curves of the 4 groups are shown in Figure 6. There was no impairment of iron absorption caused by either of the two levels of phytate as indicated by hemoglobin regeneration. In fact in the first 3 groups, final mean hemoglobin concentration in the group which received no phytate was lower than that of the other two. Although hemoglobin restoration patterns in the first three groups (diets 1, 2 and 3) were parallel, it took 5 weeks to reach the normal level, probably because the animals were larger than usual. The animals on diet 4 were smaller (Table 8) and had normal hemoglobin concentrations after 4 weeks. It appeared from these results that high levels of sodium phytate had no effect on the absorption of inorganic iron.

Fuhr and Steenbock (31) found that phytic acid did not have any effect on the amount of total body iron in

rats. However, when the level of dietary calcium was optimal, they found a reduction in the amount of hemoglobin produced. They claimed that, while this effect was not marked, it was significant, though they did not present any statistical analysis of their data. Sharpe et al. (65) found that iron absorption was impaired in human subjects when sodium phytate was added to the diet.

The highest level of phytate employed here gave a ratio of phytate to iron of about 360:1. This value is well above the ratio encountered in any of the foodstuffs tested and any possible phytate effect should have been demonstrated. Two explanations may be offered for these results. Firstly, perhaps the level of iron in the diets was sufficiently high so that, even though much of it was chelated by the phytate, there was enough left free for absorption. However, the regeneration curves (Figure 6) tend to discount this argument because, even in the phytate-free group, the restoration rate was the same as with the test diets. Secondly it is conceivable that the lack of effect on iron absorption was due to the destruction of phytate by intestinal phytase of either microfloral or tissue origin. Both of these possibilities need further study.

Effect of Calcium and Phosphorus

In Table 9 are presented the ratios of P:Fe, Ca:P and Ca:Fe in the various foodstuffs tested. There appeared to be no trend in the relationship between iron availability and any of these ratios.

The effect of calcium and phosphorus on iron utilization has been a controversial topic. Some workers reported that calcium interfered with iron absorption in anemic rats (50, 57). The levels they employed were as high as 3% calcium carbonate, a level seldom encountered in practical diets. In contrast, other workers (58, 60) found that high dietary calcium enhanced iron absorption. This has been attributed to the ability of calcium to combine with phytate and phosphate leaving less of these compounds to form insoluble iron salts (61).

From this work, it can be concluded that, under the conditions used, none of the most frequently cited factors said to inhibit iron utilization showed a consistent adverse effect on iron availability.

SUMMARY AND CONCLUSIONS

A technique was developed to study the physiological availability of food iron. The experimental work consisted of the following phases:

1. Development of a low-iron diet which could induce severe anemia and promote normal growth in young rats.
2. Testing of diets which contained iron from various sources in anemic rats and following relative hemoglobin regeneration rates.
3. Study of the effect of phytate, Ca:Fe, Ca:P, and P:Fe on iron utilization.

Three experiments were performed in which seven foodstuffs commonly consumed in the Middle East were tested. Of the three foodstuffs tested in Experiment I, kidney bean was the poorest source of iron. The availability of iron in lentil was comparable with that in broad bean. In Experiment II, chick pea proved to be a much inferior source of iron compared to ferrous sulfate. In the third experiment, okra, rice and wheat were tested. Okra and rice failed to promote hemoglobin regeneration; wheat compared favorably to iron from ferrous sulfate.

Assuming a value of 100% availability for ferrous

sulfate, the physiological availability of iron in the foodstuffs tested were 75.6, 58.8, 51.0, 1.4, 78.2, 53.5 and 86.8 for broad bean, chick pea, kidney bean, okra, peeled lentil, rice plus ferrous sulfate and wheat when availability was expressed in terms of gain in hemoglobin concentration per mg iron. The corresponding values for availability in terms of gain in total hemoglobin per mg iron were 100.6, 76.2, 84.4, 2.8, 101.9, 59.7 and 80.1 respectively.

It was concluded that:

1. The technique described is adequate for the estimation of iron availability.
2. The iron in broad bean, peeled lentil and wheat proved to be highly available. Okra and rice showed poor availability, whereas chick pea and kidney bean were intermediate.
3. High levels of sodium phytate had no effect on the utilization of inorganic iron.
4. There was no linear relationship between physiological availability of iron in the foodstuffs tested and P:Fe, Ca:P and Ca:Fe ratios.

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