U. S. DEPARTMENT OF AGRICULTURE
AGRICULTURAL RESEARCH SERVICE
RESEARCH PROGRESS REPORT
AGRICULTURAL RESEARCH, P.L. 85-934 and P.L. 89-106

1. GRANT NO. 12-14-100-9918 (61)
2. REPORT NO. 10
3. PROJECT NO.

4. FROM (Name and address of grantee)
Elaine Ranker Monsen, Ph.D.
University of Washington

5. REPORT PERIOD (Should coincide with Fiscal Report requirements)
FROM 4/15/74
TO 10/15/74

6. PROJECT TITLE
Iron Availability

7. SIGNIFICANT FINDINGS
1. Development and evaluation of an extrinsic radioactive iron tag as a model to monitor absorption of non-heme iron in both humans and experimental animals.
2. Investigation of the effect of meat in enhancing absorption of non-heme iron.
3. Examination of the effects of other dietary factors on the absorption on non-heme iron.
4. Comparative evaluation of the bioavailability of iron supplements in both humans and experimental animals.

8. SUMMARY OF PROGRESS (Give concise summary of progress for this report period.) (If additional space is required, use ARS FORM 52A)

The grant funds have supported a large body of research on iron absorption. The work has been divided into 3 areas:

A. Evaluation of an extrinsic iron tag in monitoring absorption of non-heme iron.

Comparison of the absorption of biosynthetically and extrinsically labeled iron in various plant foods has validated the use of extrinsic iron label in estimating non-heme iron absorption in human beings as well as in iron replete and iron deficient rats. The data has been compiled and published: investigation in humans is presented in Appendix 1 and the rat model in Appendix 2.

B. Investigation of the effects of food components on the absorption of non-heme iron.

1. Eleven studies have been made thus far in human beings. A standard food meal containing 100 grams of beef and a semi-synthetic meal formulated to match the standard meal with regard to calories, carbohydrates, protein, fat, calcium, phosphorus and iron have been employed. Utilizing these meals assessment has been made of the effects on iron absorption of:

a. The serial increase of beef in the semi-synthetic meal, Appendix 3.
b. The comparison of ten different animal proteins Appendix 4.
c. The addition of calcium phosphate salt Appendix 5.
d. The addition of 50mg of EDTA (ethylene diamine tetraacetate) Appendix 6.
e. Addition of ascorbic acid at 25, 50, and 100 mg. Appendix 7.
f. The effects of the components of the semi-synthetic meal Appendix 8.
g. The effects of substituting amino acids for the protein of the semi-synthetic and standard meals Appendix 9.

(cont'd page 2)

9. SIGNATURE OF PRINCIPAL INVESTIGATOR IN CHARGE
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h. The effect of cooked versus raw beef Appendix 10.
i. The effects of EDTA added at 6, 10, 25, 50, 100, 150 mg. Appendix 11.
j. The effects of meat when fractionated into a soluble supernatant and a residue fraction Appendix 12.
k. The effects of amino acids substitution in the semi-synthetic meal containing calcium phosphate salts Appendix 13.

2. Preliminary studies were made on the effects of lime treated corn on the absorption of iron. These studies are reported in Appendix 14. The initial data from a hemoglobin depletion repletion study indicates no differences in hemoglobin repletion between animals consuming diets of lime treated or water treated corn. However, in a preliminary extrinsic tag study animals that were iron depleted appeared to absorb the iron from water treated corn to a higher extent than they did from lime treated corn. Further, combining an iron low diet to the water treated corn tended to decrease the absorption of the iron in the iron depleted rats. This effect of the semi-synthetic diet may partially explain the lack of difference between the water treated and lime treated corn which was seen in the hemoglobin depletion repletion study in which the test corn is substituted for the cornstarch of the basal semi-synthetic diet.

C. Comparative effectiveness of various iron supplements.

The bioavailability of iron supplements was assessed in both humans and experimental animals.

1. Animals were utilized to assess availability of four supplementary iron compounds which were incorporated into infant cereals in a manner which met industrial procedures. It was observed that ferrous sulfate was absorbed at a higher rate than was reduced iron, which was absorbed at a higher rate than ferric orthophosphate, which in turn was absorbed at a higher rate than sodium iron pyrophosphate. The data has been compiled and published and will be seen in Appendix 15.

2. Human studies on the availability of supplementary iron in bread showed a similar ranking with ferrous sulfate being equivalent to reduced iron, both of which were absorbed at a much higher rate than sodium iron pyrophosphate or ferric orthophosphate. The data has been compiled and published and is found in Appendix 16.

3. The absorption of iron supplements in infant cereals and infant formulas was assessed in human infants, both ferric orthophosphate and iron pyro-phosphate were found to be absorbed at a very low rate compared to reduced iron and to ferrous sulfate. The data has been compiled and published and is found in Appendix 17. Further

Continuation sheet page 3 (Summary of Progress cont'd)

reference to the data on human infants has been reported by N. J. Smith and E. Rios, "Iron Metabolism and Iron Deficiency in Infancy and Childhood", Advances in Pediatrics, Vol. 21, pp. 239-280, 1954.

4. A cooperative study in assessing the efficiency of iron supplements in an animal model indicated that ferrous sulfate was absorbed at a higher rate than reduced iron of fine particles size, which was absorbed at a higher rate than reduced iron of coarse particle size, which in turn was absorbed at a higher rate than ferric orthophosphate. This was evaluated in dose reponse curve produced from a hemoglobin depletion repletion study. The data is reported in Appendix 18.

Absorption of fortification iron in bread[1, 2]

James D. Cook,[3] *M.D.*, *Virginia Minnich*,[4] *M.Sc.*, *Carl V. Moore*,[5] *M.D.*,
Arlette Rasmussen,[6] *Ph.D.*, *William B. Bradley*,[7] *Ph.D.*, *and Clement A. Finch*,[8] *M.D.*

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Voluntary or compulsory programs for adding inorganic iron to one or more foods have been adopted by many countries (1, 2). Because cereals most often provide the largest single source of calories and are inexpensive, they usually are selected as the vehicle for the addition: wheat, maize, or rice according to the dietary habits of the population. When wheat is selected for fortification, the amount of iron added is designed either to restore the iron lost in milling or to enrich the flour. In the United Kingdom, for instance, the iron content is restored to at least 1.65 mg/100 g of wheat flour (1), whereas in the United States (3) enough iron is added (12 mg/lb) to raise the level to that of whole wheat flour (3.5 mg/100 g); a proposed regulation would increase the amount to 40 mg Fe/lb (4). The biologic availability of the iron added seemed to be established when Steinkamp, Dubach and Moore (5), using radioiron techniques to measure absorption, found that healthy subjects absorbed an average of 4.2 to 6.5% from bread baked under commercial conditions with 12 mg elemental iron/lb added as ferrous sulfate, ferrum reductum, ferric orthophosphate, or sodium ferric pyrophosphate. Iron-deficient subjects absorbed a larger percentage, and the simultaneous administration of ascorbic acid increased iron uptake. There are ample reasons, however, for re-examining the question of biologic availability.

1) The sodium iron pyrophosphate used by Steinkamp and her associates (5) was almost certainly different from the material used by the baking and cereal industries today; it was a green powder rather than white (commercial product). Furthermore, the particle size and solubility of ferrum reductum were not determined.

2) The effectiveness of fortifying wheat with iron has been challenged on the grounds that: *a*) in order to avoid adverse effects on the flour, iron has usually been added in forms that are poorly absorbed (e.g., relatively large particle size powdered iron) (1); *b*) foods (e.g., eggs) often eaten with bread tend to diminish absorption of the added inorganic iron (1); and *c*) no beneficial effect could be detected on the hemoglobin level of a rather large population of anemic women whose entire bread supply for several months contained several different levels of added iron (6).

3) Recent evidence indicates that exogenous non-heme iron added to a vegetable or cereal food forms a common pool with the endogenous iron in that food and is absorbed to the same extent as is the endogenous iron

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[2] Supported by Department of Agriculture Grant 12-14-100-9918 (61) and by Public Health Service Research Grant HL-06242 and Training Grant AM-05130 from the National Institutes of Health, Bethesda, Maryland 20014. A portion of this work was conducted through the Clinical Research Center facility of the University of Washington with support by National Institutes of Health Grant FR-37.

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TABLE 1
Specifications of labeled iron supplements

Supplement	Label	Iron content, %	Specific activity, μCi/mg	Solubility	Particle size, μ[a]
Ferrous sulfate hydrate	^{55}Fe	22.3	1.8	100% in H_2O	3–5
Ferrous sulfate hydrate	^{59}Fe	21.8–23.1	5.7–11.4	100% in H_2O	5–10
Sodium iron pyrophosphate	^{55}Fe	15.4	1.4	100% in 1.2 M HCl	5–10
Ferric orthophosphate	^{55}Fe	28.7	1.6	100% in 1.2 M HCl	5–10
Reduced iron	^{55}Fe	99.7	1.1	100% in 1.2 M HCl	5–10

[a] Greater than 95% of particles were between the stated ranges.

(7). As the native iron in wheat has only moderate biologic availability (approximately 5% in fasting subjects) (8), any added iron would be absorbed only to the same extent.

This paper reports the results of a collaborative study to test four aspects of the absorption of radioactive iron supplements baked into dinner rolls at a level recently proposed for fortification in the United States (5.5 mg/100 g bread) (4). The studies were designed to compare various forms of iron supplements now used for fortification of bread, flour, and other cereal products, to test varying levels of iron supplementation, to compare absorption of the native iron in flour with that of the iron supplement, and finally, to study the availability of the iron supplement when taken with a regular diet.

Methods

Iron absorption tests were performed in 75 volunteer subjects in Seattle, Washington, and St. Louis, Missouri. Informed consent was obtained from all subjects prior to study. The age of the subjects ranged from 19 to 52 with a median of 23 years, and all but six of the subjects were females. None of the subjects were anemic as defined by WHO criteria (9) and apart from six individuals with mild iron deficiency as defined by a transferrin saturation below 15% (9), all subjects were otherwise healthy and free of disorders known to influence the gastrointestinal absorption of iron. On the first day of the study, measurements of packed cell volume, serum iron (10, 11) and iron-binding capacity measured either by coated charcoal (12) or a radioactive $MgCO_3$ method (13) were performed in all subjects.

Preparation of tagged rolls

Labeled iron supplements were prepared by Abbott Laboratories under specifications agreed upon by representatives of the major manufacturers of iron sources for enrichment and fortification.[9] Ferrous sulfate, ferric orthophosphate, and iron reduced by hydrogen were prepared as described previously by Steinkamp et al. (5). Sodium iron pyrophosphate was manufactured by a method furnished under a secrecy agreement to Abbott Laboratories by Stauffer Chemical Co. Recognizing that experimental batches could probably not be made that would meet all commercial specifications, the committee stipulated that the iron content, the solubility, and particle size should constitute the criteria of suitability for use in these experiments because these factors are probably most important in assimilation. Except for the particle size of the reduced iron, all values used were derived from the Food Chemical Codex. The specifications of the labeled supplements employed in the present study are summarized in Table 1. Because the quantities of tagged iron produced were too small to be sized by sieving, Abbott Laboratories devised a method of determining approximate particle size by suspending samples of the preparation in mineral oil. A drop of these suspensions was placed on a hemocytometer slide permitting comparison in the size of the particles with the size of the grid of the slide. Specifications supplied by industry for reduced iron included a Fisher number, which was interpreted in terms of 12 μ or less. The experimentally produced iron was milled to particles ranging in size from 5 to 10 μ except for a few agglomerates as large as 100 μ. Thus, the tagged reduced iron consisted of particles smaller than is commercially available. The better commercial products have a size distribution as depicted in Table 2.

All absorption tests were performed by a multiple dose administration technique in which the test dose was given on 5 to 10 successive occasions. In a majority of studies, a single test dose consisted of a 60-g dinner roll (average native iron content 0.5 mg Fe) containing 3 mg labeled iron supplement. Rolls were prepared at the American Institute of Baking and had the composition as listed in Table 3.

[9] Sterwin Chemicals, Stauffer Chemicals, Glidden-Durkee SCM, Mallinckrodt Chemical Works, and Merck & Co.

Labeled iron compounds were added after ingredient incorporation using a portion of the formula water. Tagged ferrous sulfate was mixed with sufficient nonradioactive carrier to obtain the required specific activity. However, the remaining iron supplements were prepared with the specific activity required for the study in order to avoid dilution with nonradioactive carrier.

Ingredients were mixed in a stainless steel bowl and fermentation was allowed to proceed without transfer from the bowl. The dough was punched and molded by hand and divided equally into the number of parts required for the study. Each dough piece was then further subdivided into exactly equal portions to provide the prescribed number of rolls per package. This method was adopted to guard against errors that would be caused by gradual moisture loss during make-up. All weighing was done with a tortion balance within a tolerance of ± 0.1 g.

The dough pieces were placed on aluminum foil-lined sheets for proofing and baking. When cool, the foil was folded around the rolls and the package placed in a plastic bag. Packages were frozen and shipped in Dry Ice to Seattle and St. Louis for administration.

Measurements in a whole-body counter (14) indicated the variation of ^{59}Fe activity between packages containing the total dose of radioactivity for each subject to be less than ± 2%. The analysis of iron and ^{59}Fe in the crust and inner portion of 10 individual rolls from two separate packages is shown in Table 4. The relative variability for each of these parameters was within ± 5%.

Iron absorption tests

Rolls containing either ^{55}Fe or ^{59}Fe, or both, were eaten between 7 and 9 AM following an overnight fast, and nothing further was allowed by mouth for 2 hr. The total dose of radioactivity in each of the absorption tests was 10 μCi for both ^{59}Fe and ^{55}Fe. Analysis by the manufacturer indicated that both ^{55}Fe and ^{59}Fe were greater than 99% radioisotopically pure. One set of rolls was reserved in each study for standards from which weighed aliquots were counted to determine the total dose of administered radioactivity. Radioiron absorption was measured 2 weeks following the test dose from the ^{59}Fe or ^{55}Fe activity in the subject's blood, using an estimated total blood volume based on sex, height, and weight (15). Red cell incorporation of absorbed radioiron was assumed to be 80% in all subjects (16).

Immediately following this study, one or two additional absorption tests were again performed with ^{59}Fe or ^{55}Fe administered by a multiple dose administration technique. In all subjects, one of these tests was performed with a standard reference dose of 3 mg Fe as ferrous sulfate 59 and ascorbic acid (2 moles ascorbic acid per mole Fe). Absorption of this iron was determined in blood obtained 2 weeks later from the rise in ^{59}Fe or ^{55}Fe activity over the previous level.

TABLE 2
Particle size distribution in better commercial products

Particle size distribution, % (roller analysis)	Electrolytic iron	Iron reduced by hydrogen
0–10 μ	39.51	3.15
10–20 μ	34.85	19.18
20–30 μ	15.73	15.78
30–40 μ	8.85	41.08
+40 μ	1.06	20.77

TABLE 3
Composition of rolls from the American Institute of Baking

Ingredient	Parts/100 parts flour
Flour	100.0
Yeast food	0.5
Yeast	3.0
Salt	2.0
Lard	7.0
Nonfat dry milk	4.0
Sugar	8.0
Water	60.0

TABLE 4
Variation in iron and specific activity of rolls supplemented with ferrous sulfate 59

Roll	Weight, g	Crust		Crumb	
		Iron, μg/g	Specific activity, μCi/mg	Iron, μg/g	Specific activity, μCi/mg
1	56.3	53.4	0.601	58.2	0.597
2	60.3	51.9	0.604	53.5	0.630
3	60.4	55.6	0.587	56.0	0.605
4	60.1	53.0	0.592	54.8	0.640
5	59.0	57.0	0.590	60.5	0.590
6	59.4	55.6	0.624	56.8	0.615
7	59.5	56.2	0.604	56.8	0.631
8	59.1	55.6	0.596	62.3	0.577
9	58.7	56.6	0.607	62.3	0.572
10	59.3	56.0	0.592	57.8	0.589
Mean	59.2	55.1	0.600	57.9	0.605
SD	1.2	1.7	0.011	3.0	0.024
CV, %[a]	2.0	3.1	1.8	5.2	3.9

[a] Coefficient of variation.

Double isotope counting of ^{55}Fe and ^{59}Fe was performed in Seattle either by liquid scintillation counting as described by Eakins and Brown (17) or by the separate assay of ^{59}Fe activity with a 3-inch sodium iodide well-type scintillation counter and ^{55}Fe activity with a proportional counter designed for direct measurement of blood ^{55}Fe (18).

In St. Louis, double isotope counting was done with the liquid scintillation counting method described by Katz et al. (19). Sufficient counts were obtained on duplicate samples to reduce the net counting rate error to less than ± 3% in subjects absorbing more than 1% of the test dose.

Absorption of the labeled supplements was expressed both as percentage absorption and in relation to the absorption of the standard reference dose of ferrous ascorbate in each subject. This latter method of expression provides an adjustment for differences in iron absorption due to individual variations in iron balance (20). Because of the skewed distribution of iron absorption data, both the mean percentage absorption and the ratio between supplement and ferrous ascorbate absorption were calculated on the logarithmic scale and retransformed as antilogarithms to recover the original units (21).

Results

Comparison of different iron supplements

The initial study concerned the availability of four types of iron supplements. Because it was thought that ferrous sulfate would be most assimilable of the various supplements, in each subject comparisons were made of absorption from rolls tagged simultaneously with ferrous sulfate 59 and one of the three

TABLE 5
Absorption of various radioiron supplements baked into rolls

Subject	Sex and age	Hct, %	Serum iron, μg/100 ml	Transferrin saturation, %	Administration	Iron absorption, %			Absorption ratios		
						A Ferrous sulfate	B Alternate	R Reference	A/R	B/R	B/A
A. Alternate, sodium iron pyrophosphate											
1 JW	F21	42	116	38	Combined	4.1	0.1	38.4	0.11	0.00	0.02
2 HH	F20	41	44	13	Combined	6.1	0.2	38.6	0.16	0.01	0.03
3 ZH	F19	43	94	22	Combined	16.0	0.3	40.5	0.40	0.01	0.02
4 JH	F26	43	88	32	Combined	24.6	0.5	90.8	0.27	0.01	0.02
5 DMc	F44	40	120	37	Separate	0.9	0.1	6.3	0.14	0.02	0.11
6 MM	F28	43	67	17	Separate	28.8	2.4	24.4	1.18	0.10	0.08
7 JB	F22	44	96	25	Separate	6.3	0.6	14.4	0.44	0.04	0.10
8 JN	F24	43	187	38	Separate	2.3	0.3	2.1	1.10	0.14	0.13
Mean[a]		42	102	28		6.6	0.3	20.0	0.33	0.02	0.05
B. Alternate, ferric orthophosphate											
1 KH	F22	39	172	53	Combined	10.9	4.0	88.3	0.12	0.05	0.37
2 MM	F19	43	103	25	Combined	2.9	0.8	18.5	0.16	0.04	0.28
3 SD	F29	42	143	40	Combined	1.5	0.6	7.1	0.21	0.08	0.40
4 CS	F21	41	92	31	Combined	11.5	4.1	40.1	0.29	0.10	0.36
5 AC	F39	43	100	24	Separate	2.1	0.6	4.4	0.48	0.14	0.29
6 EI	M19	50	146	39	Separate	3.3	1.4	18.8	0.18	0.07	0.42
7 GS	M29	47	100	26	Separate	3.1	0.9	10.4	0.30	0.09	0.29
8 BS	F28	39	204	39	Separate	2.4	0.4	13.3	0.18	0.03	0.17
Mean[a]		43	133	35		3.6	1.1	16.4	0.22	0.07	0.31
C. Alternate, reduced iron											
1 RR	F22	42	142	39	Combined	4.0	4.1	30.9	0.13	0.13	1.03
2 NM	F20	40	81	22	Combined	5.5	5.0	40.5	0.14	0.12	0.91
3 JB	F19	43	68	22	Combined	16.6	15.3	34.8	0.48	0.44	0.92
4 TN	F29	41	65	20	Combined	48.9	45.8	100.4	0.49	0.46	0.94
5 LI	F49	40	63	15	Separate	21.5	23.9	73.5	0.29	0.33	1.11
6 IR	F22	42	89	25	Separate	5.9	5.3	24.9	0.24	0.21	0.90
7 CS	F23	41	72	26	Separate	6.6	6.4	16.3	0.40	0.39	0.97
8 NF	F24	43	139	36	Separate	3.1	2.6	15.4	0.20	0.17	0.84
Mean[a]		42	90	26		9.1	8.6	34.5	0.26	0.25	0.95

[a] Geometric means have been calculated for iron absorption and absorption ratio values.

remaining supplements labeled with ^{55}Fe. Absorption tests were performed in a total of 24 subjects, using 8 subjects to compare each of the three iron supplements with ferrous sulfate. In one-half these studies, the two iron supplements, each containing 1.5 mg elemental iron, were combined into the same 60-g roll and administered on 5 successive mornings. To eliminate the possibility of interaction of the two supplements during the baking process, the remaining subjects were given two 30-g rolls, each containing 1.5 mg elemental iron and tagged separately with ferrous sulfate and the alternate iron supplement. The two rolls were again administered on 5 successive mornings. Two weeks later, a final absorption test was performed in all subjects from a standard reference dose of 3 mg ferrous ascorbate.

The results are summarized in Table 5 and Fig. 1. The least available of the iron supplements was sodium iron pyrophosphate with a geometric mean absorption of 0.3% and a mean absorption ratio relative to ferrous sulfate of 0.05. Although a somewhat lower absorption ratio was observed when this supplement was combined with ferrous sulfate in the same roll (0.02) than when rolls were separately tagged with the two iron supplements (0.10), this difference is less definite because of the extremely low assimilability of the sodium iron pyrophosphate (absorption less than 1% in all but one of the eight subjects).

Somewhat higher availability was observed with ferric orthophosphate as indicated by a mean absorption ratio of 0.31 relative to ferrous sulfate and a mean percentage absorption of 1.1%. Statistical analysis indicated that ferric orthophosphate was significantly more available than sodium iron pyrophosphate and significantly less available than ferrous sulfate ($P < 0.001$).

The highest availability relative to ferrous sulfate was observed with iron reduced by hydrogen, which had a mean absorption of 8.6% and a mean absorption ratio of 0.95 relative to ferrous sulfate. The availability of iron reduced by hydrogen was therefore significantly higher than that of ferric orthophosphate ($P < 0.001$) but not different from that of ferrous sulfate ($P > 0.10$).

Absorption of the labeled ferrous sulfate

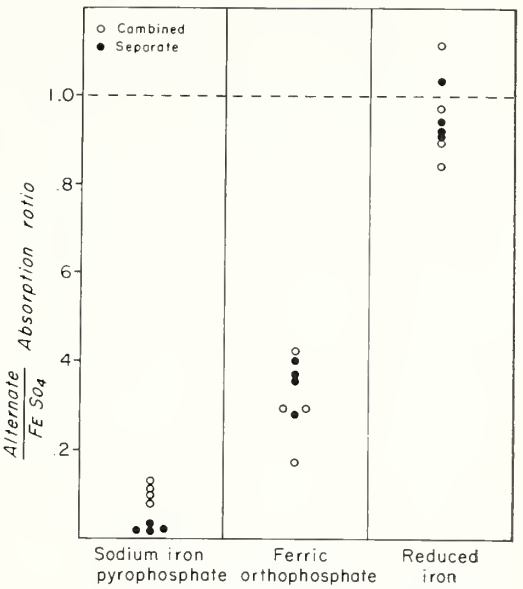

FIG. 1. Absorption of various iron supplements baked into dinner rolls. The absorption of sodium iron pyrophosphate, ferric orthophosphate, and reduced iron (alternate iron supplements) relative to ferrous sulfate is plotted. The alternate supplement and ferrous sulfate, each containing 1.5 mg elemental iron, were either baked into the same 60-g roll (combined) or baked into separate 30-g rolls and administered simultaneously (separate).

in these studies varied widely from 0.9 to 48.9% with absorption means in the various studies of 6.6, 3.6, and 9.1%. A similarly wide variation in the absorption of the reference dose of ferrous ascorbate was observed with values ranging from 2.1 to 100.4% and means in the individual studies of 16.4, 20.0 and 34.5%. By relating absorption of the ferrous sulfate supplement to the reference dose absorption within each subject, a more uniform index of the availability of the supplement is obtained. This mean absorption ratio was 0.33, 0.22, and 0.26 in the three separate studies with an overall mean of 0.26. The absorption of ferrous sulfate baked into rolls was therefore roughly one-fourth of the level observed when ferrous sulfate was given as a solution of inorganic iron.

Absorption at varying levels of iron supplementation

The next study was undertaken to determine the absorption from baked dinner rolls (60 g) of varying levels of iron supplement as

ferrous sulfate. Four iron absorption tests were performed in each of the subjects. In the first pair of tests, the subjects were given rolls supplemented with 1 mg ferrous sulfate 55 on alternate days over a 10-day period, whereas on the intervening days, they were given rolls supplemented with 5 mg ferrous sulfate 59. Fourteen days following the final test dose, a second pair of absorption studies were again performed by administering rolls tagged with 3 mg ferrous sulfate 59 on 5 alternate days and a 3-mg reference dose of ferrous ascorbate 59 on intervening days.

This protocol was used to assess absorption at varying levels of supplementation when the rolls were either taken alone or with a regular diet. Thus, in the first group of nine subjects, a 60-g roll supplemented with 1, 3, or 5 mg iron as ferrous sulfate was eaten alone as the first meal of the day for 5 alternate days. In the second group of nine subjects, two 60-g rolls baked with 1, 3, or 5 mg iron as ferrous sulfate were eaten with the regular diet each day, one with lunch and the other with dinner, for 5 alternate days.

The results are listed for each subject individually in Table 6. In the first study when the rolls were taken alone, mean absorption decreased progressively from 8.7 to 4.0% with an increase in iron supplementation from 1 to 5 mg Fe. However, absolute iron absorption increased with increasing supplementation, means of 0.087, 0.15, and 0.20 mg Fe being absorbed from rolls containing 1, 3, and 5 mg Fe, respectively. These variations were not observed when rolls were consumed with the regular diet. Thus, mean absorption from the 1-mg roll was 5.6%, only slightly higher than the mean absorptions of 5.0% observed for rolls supplemented with either 3 or 5 mg Fe as ferrous sulfate. The effect of the three different levels of supplementation observed when the rolls were taken alone was presumably masked by the iron naturally present in the self-selected meals.

Absorption of native wheat iron and supplemental ferrous sulfate

In the third study, dinner rolls were baked with wheat tagged biosynthetically by adding ^{55}Fe to hydroponic culture media (20) and with iron supplement added as ferrous sulfate 59. The iron content of the 60-g roll was

TABLE 6
Radioiron absorption from rolls supplemented with varying levels of tagged ferrous sulfate and administered either alone or with meals

	Subject	Sex and age	Hct, %	Serum iron, μg/100 ml	Transferrin saturation, %	Iron absorption, %			
						1 mg	3 mg	5 mg	Reference
Alone	1 AB	F20	37	48	10	13.9	6.8	6.5	65.6
	2 KI	F20	40	111	34	10.8	6.0	4.8	26.3
	3 KM	F19	41	102	26	4.0	1.4	1.8	10.5
	4 BM	F23	39	164	46	2.8	1.4	1.3	19.0
	5 FP	F28	44	89	27	12.9	9.8	4.5	39.3
	6 MJ	F18	43	118	35	6.8	4.8	3.9	16.9
	7 AC	F21	39	87	28	10.8	8.6	4.3	52.8
	8 CW	F21	44	81	23	21.4	11.4	12.5	45.3
Mean[a]			41	100	29	8.7	5.0	4.0	29.3
With meals	1 MS	F23	43	177	33	1.4	4.4	2.1	12.6
	2 MA	F34	42	97	37	8.5	11.9	6.6	21.3
	3 LC	M29	41	166	70	9.4	5.3	8.0	21.0
	4 BN	F19	38	123	51	13.8	4.9	8.0	32.0
	5 NB	M19	45	169	32	2.4	4.5	2.5	9.3
	6 NMc	F19	42	159	31	6.3	2.9	8.6	7.1
	7 JF	F39	42	144	28	5.0	3.1	3.5	16.1
	8 JB	M19	44	198	40	8.1	7.0	5.5	12.6
Mean[a]			42	154	40	5.6	5.0	5.0	14.9

[a] Geometric means have been calculated for iron absorption tests.

TABLE 7
Radioiron absorption from rolls baked with hydroponically tagged wheat and labeled ferrous sulfate supplement

Subject	Sex and age	Hct, %	Serum iron, μg/100 ml	Transferrin saturation, %	Iron absorption, %			Absorption ratios		
					A Ferrous sulfate	B Wheat iron	R Reference	A/R	B/R	B/A
1 RC	F 29	40	95	19	1.9	2.3	4.9	0.38	0.46	1.21
2 AH	F 34	38	54	13	6.6	7.8	19.1	0.35	0.40	1.18
3 AR	F 34	39	151	46	4.1	5.6	15.5	0.26	0.36	1.37
4 KS	F 20	38	192	40	3.4	4.1	18.6	0.18	0.22	1.21
5 LT	F 25	42	100	44	1.1	1.6	10.1	0.11	0.16	1.45
6 RS	F 34	40	70	19	9.5	11.8	62.9	0.15	0.19	1.24
7 EG	F 23	40	126	39	10.9	12.9	25.6	0.42	0.50	1.18
8 ID	F 37	38	166	37	9.9	11.6	29.8	0.33	0.39	1.17
9 BK	F 47	39	77	33	8.5	10.4	18.5	0.46	0.56	1.22
Mean[a]		39	114	32	4.9	6.1	18.4	0.27	0.33	1.24

[a] Geometric means have been calculated for iron absorption and absorption ratio values.

0.9 mg, of which one-half represented the native iron of the flour and the remaining one-half, the added iron supplement. One roll was fed after an overnight fast to each of nine subjects on 5 successive mornings. Fourteen days following the final test dose, the usual 3-mg reference dose of ferrous ascorbate 59 was given to fasting subjects for 5 successive mornings.

The results are shown in Table 7. Geometric mean absorption was 4.9% from the ferrous sulfate supplement and 6.1% from the biosynthetically tagged wheat. The mean absorption ratio of native wheat iron to the supplement iron was therefore slightly greater than unity (1.24). The mean absorption ratio of the ferrous sulfate supplement to the reference dose of ferrous ascorbate was 0.27, almost identical with the mean of 0.26 observed in study 1.

Absorption of supplemented rolls administered with a complete meal

The final study was designed to estimate the availability of ferrous sulfate supplement in baked dinner rolls when taken with a complete meal or with a regular diet. In Seattle, three types of meals were prepared in advance and frozen. The composition of these meals calculated from food tables (22) are listed in Table 8. The meals were designed so as to contain comparable amounts of calories, protein, and iron. A protein that is known to enhance the absorption of nonheme iron in the diet (25) was included in meal A (beef) but not in meals B and C. Each of the meals included a 60-g dinner roll supplemented with 3 mg iron as ferrous sulfate 55, and the identical meal was consumed on 5 successive mornings. Immediately following each meal, 0.1 mg iron as ferric chloride 59 was taken in a small volume of water to determine the absorption of the total non-heme iron in the complete meal (7). In St. Louis, absorption of iron-supplemented rolls was studied when administered with the regular diet. One roll baked with 3 mg iron as ferrous sulfate 55 plus an extrinsic tag of 0.1 mg iron as ferric chloride 59 was taken with each of the two main meals of the day for 5 successive days. Eight of the subjects were advised to eat their normal diet, whereas the remaining four subjects were asked to avoid meat, fish, and fruit. In all subjects, absorption was measured 2 weeks following the final test dose from a reference dose of ferrous ascorbate administered on 5 successive mornings.

The results in individual subjects are tabulated in Table 9; subjects are divided into those consuming meals either with or without meat protein. Of seven subjects ingesting meal A which contained 60 g beef, absorption of the ferrous sulfate supplement varied from 2.3 to 20.9%, with a mean of 5.1%. Somewhat higher absorption was observed when the rolls were taken with the regular

TABLE 8
Composition of meals used to study absorption of supplemented rolls

Item	Weight, g	Food energy, kcal	Protein, g	Iron, mg	Ascorbic acid, mg
Meal A					
Beef, flank steak	60	118	18.3	2.3	0
Potatoes	30	163	1.9	0.5	40
Green beans	65	16	0.9	1.0	4
Italian dressing	15	83	0.0	0.0	0
Cola beverage	200	78	0.0	0.0	0
Coffee	150	1	0.0	0.1	0
Roll	60	188	5.9	3.5	0
Total	580	647	27.0	7.4[a]	44
Meal B					
Liverwurst	35	112	5.2	2.1	0
Cheese	30	111	7.0	0.3	0
Vegetable juice	200	42	1.4	1.8	32[b]
Butter	7	50	0.0	0.0	0
Milk	250	108	10.7	0.2	2
Coffee	150	1	0.0	0.1	0
Roll	60	188	5.9	3.5	0
Total	732	612	30.2	8.0[a]	34
Meal C					
Eggs	100	163	12.9	2.3	0
Almonds	21	132	3.9	1.0	0
Raisins	28	81	0.7	1.0	0
Milk	250	86	8.6	0.0	2
Coffee	150	1	0.0	0.1	0
Rolls	60	188	5.9	3.5	0
Total	601	651	32.0	7.9[a]	2

[a] Iron content measured colorimetrically following wet digestion (23) was 8.2, 8.1, and 6.7 for meals A, B, and C, respectively. [b] Contained 26.8 mg ascorbic acid (24) by actual measurement.

diet (2.3 to 33.0, mean 9.6%). However, the mean ratio of ferrous sulfate absorption to the reference dose absorption was similar in subjects taking meal A (0.24) and those taking a regular diet (0.31). For the 15 subjects taking rolls with meals containing meat, the composite mean absorption of the ferrous sulfate supplement was 7.1% and the absorption ratio relative to the reference dose was 0.27.

Absorption of the supplemented rolls was less when taken with meals containing no meat protein. Thus, absorption with meal B averaged 1.8%, meal C, 1.3%, and with a full diet, 3.8%. The composite mean absorption in this group was 2.1%, and the absorption ratio relative to the reference dose was 0.11.

Absorption of the extrinsic tag of ferric chloride was similar to the absorption of ferrous sulfate supplement both in meals with and without meat protein. Thus, the mean absorption ratio of the extrinsic tag to supplement was 1.09 in meals with meat protein and 1.43 in meals without meat protein, giving a composite mean ratio of 1.22.

Discussion

Of the various methods that have been used to assess the availability of dietary iron supplements, isotope measurements are generally regarded to be the most precise. However, even with isotope studies, the experimental design is critical. For example, when single absorption studies are made in separate groups of subjects, appreciable differences in availability may be masked by the large variations in absorption among different subjects. The sensitivity of isotopic measurements can be enhanced by relating absorption of a labeled supplement to that of a standard reference dose of inorganic radioiron administered later to the same subject. Although this approach "reads out" subject-to-subject variation, there still remains a significant variability in the absorption ratio because of the day-to-day variations in assimilation by the same subject. This latter variation can be reduced but not eliminated by administering multiple test doses rather than a single dose. The most precise method for assessing availability is to compare absorption of two supplements tagged separately with ^{55}Fe and ^{59}Fe and either baked into the same roll or into separate rolls and administered simultaneously. The advantage of simultaneous administration is shown in Fig. 1 in which a highly constant ratio is seen between ferrous sulfate and each of the remaining iron supplements. A possible disadvantage of simultaneous administration is isotopic exchange between different forms of radioiron either during preparation of the test meal (e.g., baking in the present study) or in the intestinal tract following administration. The former possibility was excluded in the

TABLE 9

Radioiron absorption from rolls supplemented with 3 mg iron as tagged ferrous sulfate and administered with meals

	Subject	Sex and age	Hct, %	Serum iron, μg/100 ml	Transferrin saturation, %	Iron absorption, %			Absorption ratios		
						A $FeSO_4$ supplement	B Extrinsic tag	R Reference	A/R	B/R	B/A
With meat protein											
Meal A	MA	F23	42	92	34	2.3	2.5	12.5	0.18	0.20	1.09
	BM	F25	40	91	32	2.9	4.3	6.3	0.46	0.68	1.48
	KM	F25	40	60	23	3.4	3.6	14.5	0.23	0.25	1.06
	PD	F21	41	77	28	4.6	9.4	20.9	0.22	0.45	2.04
	MD	F21	42	51	15	6.3	9.6	58.5	0.11	0.17	1.57
	CM	F24	41	107	32	6.3	7.6	30.3	0.21	0.25	1.21
	KE	F25	45	124	31	20.9	26.4	51.7	0.40	0.51	1.26
Regular diet	MP	F33	38	98	26	2.3	2.3	9.6	0.23	0.23	1.00
	ES	F52	44	103	40	3.6	2.6	24.9	0.14	0.10	0.72
	KJ	F30	42	56	20	8.0	9.4	26.9	0.30	0.35	1.17
	BF	F23	37	89	32	12.3	9.8	41.1	0.30	0.24	0.80
	JC	F25	41	39	11	12.6	12.0	20.1	0.63	0.60	0.95
	CM	F24	38	129	34	14.4	13.1	43.0	0.33	0.30	0.91
	SF	F22	40	167	41	15.0	11.6	55.4	0.27	0.21	0.78
	PC	F22	39	103	40	33.0	31.3	63.6	0.52	0.49	0.95
Mean[a]			41	92	29	7.1	7.7	26.1	0.27	0.30	1.09
No meat protein											
Meal B	NM	F21	37	89	32	1.1	1.2	12.0	0.09	0.10	1.10
	MB	F20	42	98	34	2.0	4.1	38.4	0.05	0.11	2.05
	SK	F28	38	58	18	2.6	5.7	54.5	0.05	0.10	2.19
Meal C	NJ	F24	42	77	28	0.5	1.5	17.1	0.03	0.09	3.00
	MG	F25	43	177	51	0.9	1.5	14.8	0.06	0.10	1.67
	JB	F23	42	122	28	1.3	2.3	20.1	0.06	0.11	1.77
	NF	F25	42	75	26	5.5	7.5	34.8	0.16	0.22	1.36
Regular diet	JM	F31	44	192	43	1.3	1.5	8.8	0.14	0.17	1.20
	TF	F24	39	53	13	1.4	1.3	11.1	0.12	0.11	0.91
	PV	M23	45	129	37	6.6	5.4	11.3	0.59	0.48	0.81
	SD	F21	42	153	41	16.5	15.6	27.3	0.60	0.57	0.95
Mean[a]			41	111	32	2.1	3.0	19.2	0.11	0.15	1.43

[a] Geometric means have been calculated for iron absorption and absorption ratio values.

present study for both ferric orthophosphate and reduced iron by the similarity in results obtained with doubly tagged rolls and rolls labeled separately and administered simultaneously. Isotopic exchange between ^{55}Fe and ^{59}Fe-tagged supplements following administration would minimize rather than accentuate any differences in absorption between ferrous sulfate and the alternate iron supplements.

It is of particular interest in the present study that the absorption of small particle size ferrum reductum baked into rolls was approximately the same as from ferrous sulfate supplemental rolls. Whether solubilization occurred during fermentation of the dough, during baking, or after ingestion was not determined, but as absorption was equivalent to that of wheat iron, it probably also became part of the non-heme iron pool. The results emphasize the importance of strict definition of the properties of ferrum reductum used for enrichment of flour; reduced iron of larger particle size and certainly powdered iron is of low biologic availability (1, 26). It should therefore be stressed that commercially available reduced iron may have a lower assimilability than that found in these experiments. Until evidence to the contrary is produced, caution must be used in interpreting the results obtained in this study in terms of commercially reduced or electrolytic

iron. The poor absorption from sodium iron pyrophosphate- or ferric orthophosphate-supplemented rolls indicates that these iron preparations are not satisfactory supplements. Why absorption was so poor as compared with previous results (5) is not clear, but the experimental design was less accurate, and the preparations used may well have been different from those in the current studies. It should also be noted that comparisons in availability of different supplements were made in the present study with test doses containing only rolls rather than a normal meal. It is possible that even greater differences would be observed when rolls are administered with a complete meal.

Because ferrous sulfate appeared to be an optimal form of iron for supplementation, further studies were carried out to evaluate its effectiveness when administered with food. Composite data on availability of ferrous sulfate fortification are shown in Fig. 2. In a total of 32 fasting subjects given a 60-g roll containing tagged ferrous sulfate and 3 mg

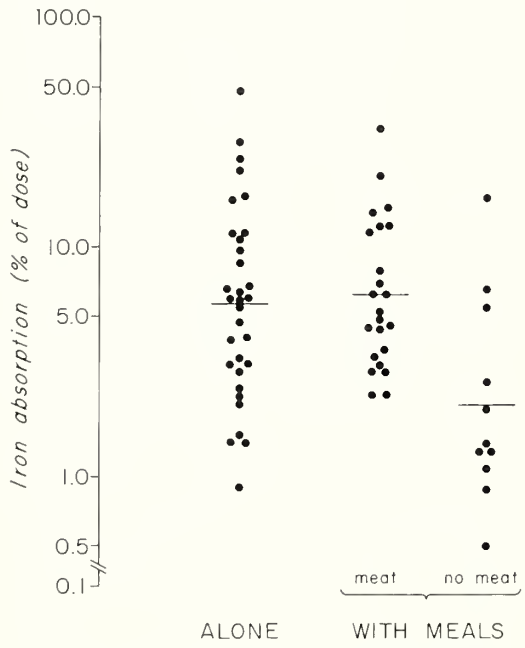

FIG. 2. Absorption from a 60-g dinner roll of 3 mg iron supplement as tagged ferrous sulfate. Rolls were either administered to fasting subjects (alone) or taken with meals with or without meat protein. Percentage absorption is plotted on a logarithmic scale. The horizontal bars depict the geometric mean absorption.

supplemental iron, the geometric mean absorption was 5.7% (± 1 SD, 2.2 to 15.0%). The mean absorption ratio of the supplement to the reference dose of 3 mg ferrous ascorbate in these subjects was 0.24 (± 1 SD, 0.14 to 0.45), which is remarkably similar to the ratio of 0.29 previously observed with biosynthetically tagged wheat (9, 27). Thus, the absorption of this iron salt was reduced by the presence of the roll to one-fourth of its absorption when given alone. When taken in a larger meal, this absorption was maintained provided meat was present in the meal. However, in the absence of meat, there was a further reduction to approximately one-tenth of the absorption from the iron salt alone.

Absorption from 60-g rolls supplemented with 1, 3, and 5 mg of elemental iron added as ferrous sulfate showed the expected reduction in percent absorption although total absorption was increased (Table 6). However, when the rolls were fed with each of the two main meals of the day, this relationship was no longer evident, presumably because the non-heme iron in the meal contributed enough iron to mask the effect of the three different levels of supplementation.

Absorption from rolls supplemented with 3 mg iron as ferrous sulfate administered alone to fasting subjects was similar to absorption when given with a regular diet containing meat protein. In 16 subjects (studies 2 and 4), one roll was taken with each of the two main meals of the day for 5 successive days, whereas in the remaining 7 subjects, one roll was taken on 5 successive days with a meal considered to represent a typical American meal. In this composite group of 23 subjects, the geometric mean absorption was 6.3% (± 1 SD, 3.0 to 13.1%), and the mean absorption ratio relative to the reference dose was 0.29 (+ 1 SD, 0.18 to 0.47). In contrast, when rolls were ingested with meals that contained no meat protein, the geometric mean absorption fell to 2.1% (± 1 SD, 0.8 to 5.8%) and the mean absorption ratio relative to the reference dose decreased to a mean of 0.11.

It is concluded that when rolls are supplemented with a highly available form of iron such as ferrous sulfate, absorption in normal subjects averages approximately 6% regardless of whether the rolls are ingested alone or

with a complete diet. The similarity in absorption when rolls are ingested alone as compared with a regular diet is somewhat surprising. One might anticipate a lower percentage absorption when the tagged rolls were taken with the regular diet because of the much larger amounts of iron contained in these meals. However, presumably enough meat protein was ingested with these meals to offset this effect. These studies also reemphasize the importance of the presence of meat in determining the availability of dietary iron.

These studies provided an opportunity to examine the behavior of fortification iron as compared with the non-heme iron pool in the diet. Previous studies have indicated that if a tracer dose (0.1 mg iron) of ferric chloride is added to a meal, the absorption of this iron corresponds closely to the absorption of biosynthetically tagged foods present in the same meal. Additional studies have indicated that fortification iron added as tagged ferric chloride in doses up to 60 mg elemental iron also corresponds closely in its availability to biosynthetically tagged foods in the same meal (25). In this study (Table 7), the ferrous sulfate supplement appeared slightly less well (4.9%) absorbed than a biosynthetically tagged wheat (6.1%). On the other hand, absorption of an extrinsic tag of ferric chloride closely paralleled absorption of tagged supplement in rolls administered with a complete meal (Table 9). The composite mean ratio of extrinsic tag to supplement in the meals with and without meat protein was 1.22, similar to the ratio of 1.28 previously observed between the extrinsic tag and biosynthetically labeled non-heme iron in a complete meal when the extrinsic tag was performed by the method used in the present study (0.1 mg ferric chloride taken as a drink following the meal) (7). Thus, further evidence has accrued to validate the presence of a single non-heme iron pool including both food iron and fortification iron, provided the supplemental iron is an available iron salt such as ferrous sulfate.

Summary

A collaborative study has been made of the absorption of radioiron supplements (3 mg iron) baked into 60-g dinner rolls. The labeled iron supplements (ferrous sulfate, reduced iron, ferric orthophosphate, sodium iron pyrophosphate) were prepared under specifications agreed upon by representatives of the major manufacturers of iron sources for enrichment and fortification in the United States. Particle size for each of the supplements varied between 3 and 10 μ. Using dual radioiron tags, three to four separate absorption tests were performed in each of 75 subjects by a multiple dose administration technique.

Absorption of sodium iron pyrophosphate was one-tenth and ferric orthophosphate one-third that of ferrous sulfate when baked into the same roll or into separate rolls and administered simultaneously, whereas ferrous sulfate and iron reduced by hydrogen were about equally available. In 32 fasting subjects given a 60-g roll containing 3 mg supplemental iron as tagged ferrous sulfate, absorption averaged 5.7% and was roughly one-quarter of the absorption from the same dose of ferrous sulfate administered as a solution of inorganic iron. When rolls supplemented with 3 mg iron as tagged ferrous sulfate were taken with meals or with a regular diet, absorption averaged 6.3 and 2.1% from meals with and without meat protein, respectively. Studies with an extrinsic tag demonstrated that fortification iron in the form of ferrous sulfate underwent complete exchange with the non-heme pool of dietary iron.

The authors wish to acknowledge the skillful technical assistance of Mary Elizabeth Smith and Mary J. Eng.

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Absorption of Fortification Iron by the Rat: Comparison of Type and Level of Iron Incorporated into Mixed Grain Cereal

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Under specifications agreed upon by representatives of the major U. S. A. manufacturers of iron sources for commercial enrichment and fortification, four iron supplements were prepared: ferrous sulfate; reduced iron; ferric orthophosphate; and sodium iron pyrophosphate. Relative availability of these iron supplements when incorporated into an infant cereal, in a manner meeting manufacturing procedures, was assayed in the rat. Iron was given at three dosage levels, 20, 80, and 320 μg/0.500 g of cereal. Ferrous sulfate was the most available supplement, followed by reduced iron. Ferric orthophosphate and sodium iron pyrophosphate were poorly absorbed. At the highest dosage level, considered to be beyond the normal dietary range, absorption of all supplements was poor. Current human studies show similar relative availability of the iron supplements, indicating effectiveness of the animal assay. Obligatory assessment of the availability of iron supplements should be an integral part in guidelines for food fortification.

Earlier studies by Steinkamp, Dubach, and Moore on the absorption of iron from iron-enriched bread led to the assumption that there were no significant differences in the biological availability of ferrous sulfate, reduced iron, ferric orthophosphate, and sodium ferric pyrophosphate (Steinkamp *et al.*, 1955). However, this assumption has been challenged as fortification programs utilizing these salts have not been uniformly effective (Elwood, 1968). Further, there are reasons to believe that the chemical and physical specifications of the iron compounds used by Steinkamp *et al.* differ markedly from those used by the food industry today (Cook *et al.*, 1973). Thus, as increasing attention is being given to the need for additional iron fortification, it is especially important to reexamine the assimilability of various iron forms suitable for commercial usage (Elwood, 1968; Finch and Monsen, 1972; Monsen *et al.*, 1967).

The purpose of this particular study was to test in an animal system the availability of various iron supplements after incorporation into a commercially prepared mixed grain infant cereal. Four iron compounds were assessed: reduced iron; sodium iron pyrophosphate; ferric orthophosphate; and ferrous sulfate. Commercial specifications were met in both the preparation of the iron supplements and their subsequent incorporation into the cereal.

METHODS AND MATERIALS

Experimental Animals. Rats were Sprague-Dawley weanling males, 3 weeks of age, weighing 45 to 50 g. They were maintained in the laboratory for 2 weeks prior to receiving the test meal in order to standardize living conditions and iron nutriture. Animals were individually housed in galvanized expanded metal cages. Weights of rats were taken on alternative days throughout the stabilization period. Laboratory chow (Ralston Purina Co., St. Louis, Mo.), containing at least 23% protein and 0.02% iron as ferric ammonium citrate and iron oxide, was fed *ad libitum* to the animals except during fasting and administration of the test meals. Water of negligible iron content was available at all times.

Approximately 4 days before test feeding, the animals were transferred to individual plastic cages to eliminate possible iron contamination during feeding. All rats were test fed on the 34th, 35th, or 36th postnatal day. A group of 8–10 animals was used for each treatment, with a total of 12 experimental groups. At the end of the period, hematocrits were assessed utilizing microcapillary tubes. At the time the test meals were administered, the body weight of the animals was 139 ± 8 g (mean ±SD).

Iron-Fortified Cereals. Mixed grain infant cereal was prepared and supplied by the Gerber Co., Fremont, Mich. The cereal was composed of oat flour, soft wheat flour, corn flour, barley flour, calcium phosphate (di-basic), barley malt flour, niacinamide, thiamine mononitrate, and riboflavin. The nutrient content of the cereal without iron supplementation was as follows: protein, 11.7%; fat, 4.5%; available carbohydrates, 73.0%; crude fiber, 1.1%; moisture, 7.0%; ash, 2.7%, calcium, 0.53%, phosphorus, 0.66%, and iron, 0.004%.

The mixed grain cereal was available in three forms: without iron; fortified with ^{59}Fe supplements; and fortified with nonradioactive iron supplements. The various ^{59}Fe compounds were incorporated in a manner similar to the commercial method for fabricating the cereal, which includes heating, enzymatic action, and drying after fortification. All labeled iron compounds utilized for fortification had been supplied to the cereal manufacturer by Abbott Laboratories and were prepared in a manner meeting specifications approved by a committee of representatives of the primary producers of iron compounds for enrichment and fortification (Glidden-Durkee SCM, Mallinckrodt Chemical Works, Merck and Co., Stauffer Chemicals, and Sterwin Chemicals). Sodium iron pyrophosphate was manufactured by a method furnished under a secrecy agreement to Abbott Laboratories by Stauffer Chemical Co. The particle size of each of the experimental iron supplements was similar to commercial sources with the exception of the reduced iron, which was 95% in the 5–10 μ range in the experimental product and 90% in the 25–45 μ range in the commercial material (Gerber Co., 1971). Detailed specifications are given by Cook *et al.* (1973), whose human studies employed identical iron supplements.

Test Meals. Analysis of the chemical iron content of each sample was performed utilizing the bathophenanthroline method following wet ashing (Bothwell and Finch, 1962). To achieve each of the desired fortified iron dosages and the standardized level of radioactivity of approximately 0.15 μCi/test meal, mixtures of the radioactive iron fortified, nonradioactive iron fortified, and unfortified cereals were made. Each test meal was composed of a total of 0.500 g of cereal mixed with 1.2 ml of noniron supplemented infant formula (Ross Laboratories, Columbus, Ohio) which had been diluted by half with deionized water to the concentration normally fed to infants. The natural iron content of the cereal, milk, and water in each test dose approximated 18 μg, with various levels of forti-

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Reprinted from AGRICULTURAL AND FOOD CHEMISTRY, Vol. 21, No. 5, Page 913, Sept./Oct. 1973

Table I. Summary of Results of a Study of the Absorption by Rats of Four Iron Salts Incorporated into Infant Cereals at Three Different Levels. Values Reported as Mean ± SD

Iron salt	Dose, g	Number of animals	% supplemental iron absorbed	Total μg of supplemental iron absorbed	Body weight, g	Hematocrit, %	Weight gain, g/day
Ferrous sulfate	20	10	15.7 ± 4.8	3.1 ± 1.0	146.9 ± 9.0	41.1 ± 1.0	6.9 ± 0.6
	80	6	12.0 ± 3.2	9.6 ± 2.6	130.6 ± 7.0	41.1 ± 2.2	6.8 ± 0.5
	320	7	3.8 ± 1.4	12.2 ± 4.5	139.7 ± 7.2	41.3 ± 3.4	6.8 ± 0.3
Reduced iron	20	6	6.7 ± 2.8	1.3 ± 0.6	129.3 ± 10.5	43.6 ± 4.5	6.1 ± 0.8
	80	7	5.1 ± 2.0	4.1 ± 1.6	135.7 ± 16.0	40.7 ± 3.0	6.4 ± 0.7
	320	7	4.6 ± 1.7	14.7 ± 5.4	138.3 ± 14.6	43.3 ± 2.9	6.2 ± 0.5
Ferric orthophosphate	20	7	2.9 ± 0.9	0.6 ± 0.2	126.8 ± 9.6	42.9 ± 4.0	5.7 ± 1.0
	80	7	3.8 ± 1.2	3.0 ± 1.0	135.7 ± 8.6	41.1 ± 1.2	6.6 ± 0.5
	320	7	3.4 ± 1.5	10.9 ± 4.8	150.0 ± 7.2	41.6 ± 2.2	6.6 ± 0.6
Sodium iron pyrophosphate	20	7	1.0 ± 0.26	0.2 ± 0.1	134.7 ± 12.8	41.7 ± 2.2	6.1 ± 0.5
	80	8	1.0 ± 0.49	0.8 ± 0.4	146.4 ± 12.4	42.0 ± 1.6	6.2 ± 0.8
	320	7	0.6 ± 0.18	1.9 ± 0.6	151.1 ± 13.0	41.7 ± 1.5	5.6 ± 0.5

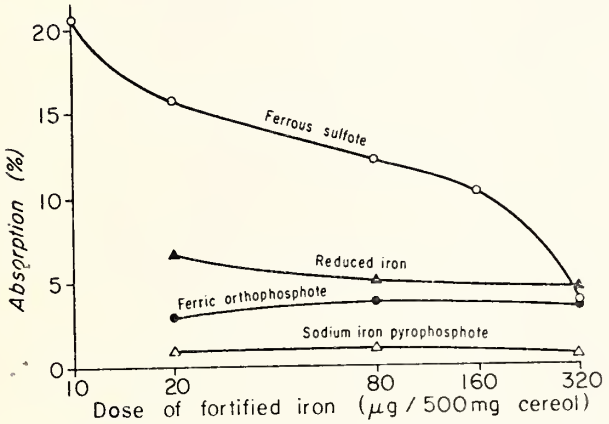

Figure 1. Absorption by rats of four iron salts incorporated into a mixed grain cereal for infants.

fied iron superimposed. Each salt was given at three levels, *viz.*, 20, 80, and 320 μg of iron/dose, as fortifying iron.

Prior to testing, rats were fasted for 16 hr; water was available during this interval. Animals were individually offered the test meal and subsequently counted in a Packard-Armac whole body counter. Normal food was replaced 2 hr after test doses had been eaten. A second total body count 7 days after ingestion of the radioactive test dose was utilized in assessing percentage absorption (Amine and Hegsted, 1970; Monsen *et al.*, 1970).

Analysis of variance (Snedecor, 1956) was performed on data for the different groups of rats and for data obtained at different dosage levels.

RESULTS

Mean values for iron absorptions, hematocrits, body weights, and weight gains for animal groups receiving each of the four salts given at the three dose levels are presented in Table I. All animals had normal hematocrits (Spector, 1956) of between 39 and 50% just prior to test feeding. The mean hematocrits of the various rat groups were not significantly different. The mean rate of growth for the 2 weeks postweaning was 6.8 g/day, normal for animals of this strain and age (Ranhotra and Johnson, 1965). No significant difference between the mean rate of growth of the various groups was observed.

The availability of the four iron salts in the mixed grain cereal is compared in Figure 1. Ferrous sulfate was the best absorbed salt when given at normal dietary levels ($p < 0.01$). The hydrogen-reduced iron tested here was found to be the next most available salt for the test animals, being absorbed 43% as well as $FeSO_4$ at the levels of 20 and 80 μg of iron/0.500 g of cereal.

Ferric orthophosphate and sodium iron pyrophosphate were poorly absorbed at all levels. Mean percentage absorption for the ferric orthophosphate at the 20-μg level was 18% of the comparable $FeSO_4$-supplemented cereal. The sodium iron pyrophosphate exhibited the poorest absorption. At the 20- and 80-μg iron level, sodium iron pyrophosphate was 6 and 8% as well absorbed as $FeSO_4$.

At the highest dietary level, 320 μg of iron/0.500 g of cereal, the mean absorptions of all salts were below 5.0%. An analysis of variance showed the absorption of $FeSO_4$ significantly declined with increased level of iron supplementation ($p < 0.01$). Differences shown in the absorption curves of the other salts were not significant.

DISCUSSION

In comparing the availability of various iron salts, both the chemical and physical characteristics of the salts play critical roles. Utilizing a short-term radioisotopic technique in rats, the present study confirms ranking of iron availability: ferrous sulfate > reduced iron > ferric orthophosphate > sodium iron pyrophosphate. This pattern appears independent of the technique employed, as much of the data reported by other investigators was obtained by assessment of hematopoiesis in the rat over a long period of time (Amine *et al.*, 1972; Blumberg and Arnold, 1947; Fritz *et al.*, 1970; Hinton and Moran, 1967; Ranhotra *et al.*, 1971). Species differences in iron absorption (Bothwell and Finch, 1962) eliminate the possibility of direct application of results with rats to human situations; however, there is evidence that the rat absorbs various forms of iron with the same predisposition to order as does man (Cook *et al.*, 1973; Elwood, 1968; Fritz *et al.*, 1970; Hoglund and Reizenstein, 1969; Layrisse *et al.*, 1968). Of particular importance are the data confirming rank order of availability issuing from the human study by Cook *et al.* (1973), which assessed the absorption of identical iron supplements manufactured to commercial specifications when these supplements were baked into rolls. As similar relationships were observed from the animal model data and the parallel human study, the further use of the animal model is recommended, especially in ways which may either decrease the need for human experimentation or pinpoint areas of critical concern for human study.

Aside from the chemical characteristics of individual iron salts, differences in the physical parameters of the specific salts themselves appear to affect absorption. Several physical forms of sodium iron pyrophosphate, ferric ammonium citrate, and reduced iron have been mentioned in studies of iron availability (Elwood, 1968; Fritz *et al.*, 1970; Hinton and Moran, 1967; Ranhotra *et al.*, 1971). Two important factors contributing to physical variation are the method of preparation and the particle size (Elwood, 1968). With regard to particle size, Hinton

et al. (1967) observed a relationship between particle size and solubility. The reduced iron used in this study was of a smaller particle size than the reduced iron used in the commercial product (Gerber Co., 1971). To the degree that larger particle size decreases availability, it would be assumed that the iron absorbed from the commercial reduced iron-supplemented product would be lessened.

It is obvious that each form of iron used for fortification should be standardized. Indeed, where inorganic iron is incorporated into food, the specific chemical and physical form of iron must be identified before one can gain an idea of its availability. Such acknowledgment is seldom given on commercial products and, even more unfortunately, such information is too frequently omitted from research reports, making comparisons impossible (Steinkamp *et al.*, 1955).

Of the iron salts which would be classed as available, an inverse relationship is seen between per cent absorption and level of fortification. Although the absolute amount of iron absorbed was greater when larger doses were ingested, it was accompanied by a decreased efficiency of utilization. This has been reported by others for rats (Bannerman, 1965; Bannerman *et al.*, 1962; Forrester *et al.*, 1962) and human infants (Garby and Sjolin, 1959; Schulz and Smith, 1958). In this study, when the dose of ferrous sulfate was increased 300% from 80 to 320 μg, only a 33% increase was observed in the absolute amount of iron absorbed. Thus, a well absorbed salt could be incorporated at a low level to furnish the iron required by an organism.

Certain aspects of iron absorption illustrated in this study should be taken into account by nutritional science and the food industry in dealing with human dietary iron problems and establishing guidelines to provide for effective food fortification. Specifically, variations in availability of various iron salts, further documented here, must be recognized. The value of an iron-containing food ought to be assessed as to its availability and not simply on the basis of total iron content. Guidelines requiring fortifying iron to be of types utilized efficiently should eliminate the addition of large amounts of unusable iron. Additionally, preparation of salts used in fortification should be standardized with regard to particle size as well as other key physical and chemical characteristics. As research yields data regarding food factors which facilitate iron absorption and verify the relative availability of various iron compounds to humans, guidelines should be revised to allow for practical implementation in terms of increasingly beneficial fortification programs.

ACKNOWLEDGMENT

The authors wish to thank Wayne J. Smalligan, Cereal Laboratory, Gerber Products Co., for cooperation in supplying the iron-fortified cereals. For her technical assistance the authors are grateful to Kerry Jacobson.

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Received for review March 5, 1973. Accepted May 29, 1973. Research supported in part by the Human Research Division, Agricultural Research Service, U. S. Department of Agriculture, Grant No. 12-14-100-9918(61).

Reprinted from THE JOURNAL OF CLINICAL INVESTIGATION, Vol. 51, No. 4, April 1972
Printed in U. S. A.

Food Iron Absorption Measured by an Extrinsic Tag

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ABSTRACT The paper describes the use of an extrinsic tag of inorganic radioiron to determine the total absorption of nonheme iron from a complete meal. The method was developed by measuring the iron absorbed from vegetable foods containing biosynthetically incorporated ^{58}Fe (intrinsic tag) and from ^{59}Fe added as a small dose of inorganic iron to the same meal (extrinsic tag). In studies with maize, black bean, and wheat, a consistent extrinsic: intrinsic radioiron absorption ratio averaging 1.10 was observed. Similar results were obtained with either ferrous or ferric iron as the extrinsic tag, and with doses of the latter ranging from 0.001 to 0.5 mg iron added to a test meal containing 2–4 mg of food iron. Adding the radioiron at different stages in preparation of the test meal also had little effect. Separate administration of the extrinsic tag was less satisfactory when small portions of a single food were employed, but with a complete meal, the separate dose was preferable. The extrinsic tag provided a valid measure of absorption despite marked differences in the iron status of the subject, and with wide changes in absorption imposed by adding desferrioxamine or ascorbic acid to the test meal. These findings indicate that there is a common pool of nonheme iron, the absorption of which is influenced by various blocking or enhancing substances present in the meal.

INTRODUCTION

The principle of employing biosynthetically tagged foods, described originally by Moore and Dubach (1) has been applied extensively in studies of iron availability from various plant and vegetable foods (2–13). Although these studies have yielded useful information, this kind of approach has serious limitations. Not only is the preparation of labeled foods difficult, but their absorption differs depending upon whether the tagged food is eaten by itself or with other foods (7, 11, 12). Thus, results obtained by biosynthetic labeling may have little bearing on the problem of iron absorption from a normal diet.

The objective of the present study was to measure the combined absorption of nonheme iron from all food sources in a regular meal. A comparison was made between the absorption of ^{55}Fe incorporated biosynthetically into the food by hydroponic culture (intrinsic tag) and a tracer dose of inorganic ^{59}Fe (extrinsic tag) administered in the same meal. The close and highly consistent relationship observed between the absorption of these two tags indicates the feasibility of assessing the absorption of nonheme iron in a normal diet by using an extrinsic label.

METHODS

Experimental subjects. Iron absorption was measured in 180 volunteer subjects from an agricultural area of Venezuela and from Seattle, Washington. Except for a high prevalence of iron deficiency, all of the subjects were otherwise healthy and free of disorders known to affect the absorption of iron. Measurements of packed cell volume, serum iron (14), and iron-binding capacity (15, 16) were performed in all subjects. The results of these tests in the 96 females and 84 males are listed individually in Tables IV and V of the Appendix and summarized in Table I.

Radioiron absorption measurements. The test meal tagged with ^{55}Fe and ^{59}Fe was eaten in the morning by subjects who had fasted overnight and were allowed no further food or drink for 3 hr. The amount of radioactivity in the food administered was determined by counting three weighed samples and extrapolating these counts to the weight of the total test meal. 2 wk later, radioiron absorption from this meal was calculated from the ^{59}Fe and ^{55}Fe activity in the subjects' blood, using an estimated total blood volume based on sex, height, and weight (17). Red cell incorporation of absorbed radioiron was assumed to be 90% in all subjects.

Immediately after this study, the subjects were again fasted and then given orally 3 mg iron as $^{59}FeSO_4$ and ascorbic acid (2 moles ascorbate per mole of iron) to provide

Received for publication 25 August 1971 and in revised form 29 November 1971.

TABLE I

Iron Status of Composite Group of 169 Volunteer Subjects

	Median	10–90 Percentile
Age, *yr*	33.0	16–55
Hematocrit, %	40.0	35.2–45.5
Serum iron, *μg/100 ml*	80.0	29–133
Transferrin saturation, %	24.0	7.2–40.6
Inorganic iron absorption*, %	35.1	6.0–80.6

* Reference dose of 3 mg ferrous ascorbate.

a reference dose. Absorption of this iron was determined in blood obtained 2 wk later from the rise in ^{59}Fe activity over the previous level.

Double isotope counting of ^{55}Fe and ^{59}Fe was performed either by liquid scintillation counting as described by Eakins and Brown (18) or Dern and Hart (19, 20), or by the separate assay of ^{59}Fe activity with a 3-inch NaI well-type scintillation counter and ^{55}Fe activity with a proportional counter recently designed for direct measurement of blood ^{55}Fe activity (21). Sufficient counts were obtained on duplicate samples to reduce the net counting rate error to less than ±2% in subjects absorbing more than 1% of the test dose.

Preparation of labeled foods. The preparation of biosynthetically tagged corn, wheat, soybean, and black bean was performed by adding ^{55}Fe to hydroponic culture media as previously described (5, 10). The harvested foods contained between 25 and 120 μg Fe/g of dry food and ranged in SA from 45 to 90 μCi/mg Fe. Labeled food was mixed with two to four times its weight of unlabeled carrier food to obtain test meals containing 10 μCi ^{55}Fe and either 2 mg elemental iron (maize and wheat) or 3 mg elemental iron (soybean and black bean).

The extrinsic tag was prepared by mixing 5 μCi $^{55}Fe Cl_2$ (SA, 10–15 mCi/mg) with varying amounts of carrier $FeCl_3$. The dose of the extrinsic tag was usually 0.1 mg iron with the exceptions noted later. The time of its administration in relation to the test meal was varied as part of the experimental design, as indicated in the appropriate sections.

Maize was prepared by boiling finely ground radioactive corn to obtain a gruel. Carrier maize was boiled separately and then ground to prepare a dough. The extrinsic tag solution was either added to the water used to boil the radioactive maize or was thoroughly homogenized with the radioactive gruel after boiling. The radioactive and carrier maize preparations were then thoroughly homogenized and divided into carefully weighed individual portions of approximately 100 g which were placed in aluminum pans and baked before administration.

Labeled black beans were boiled together with carrier beans in five to six times their weight of water in aluminum pots. The swollen beans were then mashed, mixed with the extrinsic tag solution, and cooked further before dispensing. Soybean meals were prepared as described previously (10); in this case, the extrinsic tag was taken separately in a small volume of water at the conclusion of the meal. Wheat was prepared as previously described (5), and the extrinsic tag was incorporated by adding it to the water used for making the wheat dough. Veal muscle was made into meat patties for ingestion with a test meal of labeled maize.

The validity of using an extrinsic tag to measure nonheme iron absorption from a complete meal was tested with a 700 cal standard meal of potatoes, beef, bread, margarine, peaches, milk, and hydroponically-labeled ^{55}Fe maize (Table II). This meal, estimated to have 4.5 mg elemental iron from food composition tables (22), was found to contain 4.0 mg by actual measurement. The extrinsic tag was incorporated in two ways. In the first, 0.1 mg iron as $FeCl_3$ was thoroughly mixed with the boiled maize, which was later homogenized in a Waring blendor with the remaining foods of the meal. In the alternate method, the extrinsic tag was administered separately at the end of the meal. These two methods of extrinsic tagging were tested in alternate subjects.

Statistical analysis. Food iron absorption was expressed in relation to the absorption of a standard reference dose of ferrous ascorbate in each subject. This method of expression provides an adjustment for differences in absorption due to individual variations in iron balance (10). Because of the skewed distribution of iron absorption data, the mean and standard deviation of the ratio between food iron and ferrous ascorbate absorption (absorption index) were calculated on the logarithmic scale and retransformed as antilogarithms to recover the original units (10).

The relationship between extrinsic and intrinsic radioiron absorption from the same meal (the E:I ratio) was first analyzed by least squares regression. As shown in Fig. 1, the results were consistent with a regression line fitted through the origin. The variability about the regression line with simultaneous absorption measurements was constant at increasing levels of per cent absorption. Thus, the E:I ratio could be calculated from the slope of a least squares regression line fitted through the origin (reference 23, p. 166). The standard error of the estimate was used to compare the variability of the ratios in different studies using Bartlett's test (reference 23, p. 296). In studies with homogeneous variances, differences in the E:I ratio were tested by covariance analysis (24).

RESULTS

Preliminary studies dealt with the valence of the iron employed as an extrinsic tag. In 18 studies on 6 subjects in which $FeSO_4$ was added to maize, the mean absorption index for the extrinsic tag (geometric mean ratio of extrinsic tag to reference dose absorption) was 0.19. In 15 studies on 5 subjects in which $FeCl_3$ was used,

TABLE II

Composition of Meal Used to Evaluate Extrinsic Tag

Food item	Weight	Food energy	Protein	Iron
	g	*cal*	*g*	*mg*
^{55}Fe corn	110	36	0.9	0.2
Instant potatoes	110	36	0.7	0.2
Lean beef	100	180	20.7	3.1
Bread	23	62	2.0	0.6
Margarine	20	144	0.1	0.0
Peaches	120	94	0.5	0.3
Ice milk	100	152	4.8	0.1
Total	583	704	29.7	4.5*

* The meal contained 4.0 mg iron by actual measurement.

the mean absorption index was 0.22. Trivalent iron was used in subsequent studies.

The effect of varying the dose of extrinsic tag was examined over a range from less than 0.001–0.5 mg Fe as $FeCl_3$ added to the labeled maize. The results obtained in three groups of subjects (studies 1, 2, and 3) are listed individually in Table IV of the Appendix and summarized in Table III. The mean E:I ratio in the three studies varied from 0.97 to 1.10 with an over-all mean of 1.06. An intermediate dose of 0.1 mg Fe as $FeCl_3$ was chosen for extrinsic labeling in subsequent studies.

The effect of varying the manner of adding the extrinsic tag was next examined (Table III). With test meals of maize, Fe as $FeCl_3$ was added before boiling in amounts of 0.001 mg (study 4) and 0.1 mg (study 5). In both instances, the mean E:I ratio was 1.12. When the iron was given separately, the ratios obtained were 1.43 with maize (study 6) and 1.27 with soybean (study 7). However, the variability of the ratio in individual subjects (SE of the estimate, 2.99 and 2.19) was greatly increased by this method of iron administration.

Absorption of the extrinsic tag was also examined with hydroponically labeled wheat (study 8) and black bean (study 9) by adding 0.1 mg Fe as $FeCl_3$ to boiled food before baking. The mean E:I ratio was 1.18 with wheat and 0.99 with black bean as compared with 1.06 obtained with similarly tagged maize. These differences are not statistically significant.

In studies described so far, the mean absorption of food iron (intrinsic tag) in the various groups fell within the relatively narrow range of 1.5–6.3%, with individual subjects showing variations from 0.1 to 24.4%. To examine the validity of using an extrinsic tag at extremes in food iron absorption, test doses of maize were mixed with 500 mg of either desferrioxamine[1] or ascorbic acid before administration (studies 10 and 11). Mean absorption of intrinsic tag was depressed to 0.6% with desferrioxamine, and increased to 22.0% with ascorbic acid. Nevertheless, the mean E:I ratios remained similar to those obtained previously, being 1.14 and 1.01 respectively.

The validity of the extrinsic tag for test meals containing more than a single food was then examined. The addition of veal muscle caused an increase in mean absorption of maize iron (intrinsic tag) to 9.7%. Nevertheless, the E:I ratio of 1.16 was similar to that obtained with maize alone. In the final two studies, iron absorbed from the 700 cal complete meal was measured. When extrinsic tag was given after the test meal prepared in the normal manner (study 13), the E:I ratio was 1.28. With earlier addition of the extrinsic tag and complete homogenization of the meal (study 14), the ratio was 1.06. Thus, the presence of several foods in a meal appeared to have little or no effect on the E:I ratio as compared with giving a single food (study 2).

[1] Desferal, Ciba Pharmaceutical Co., Summit, N. J.

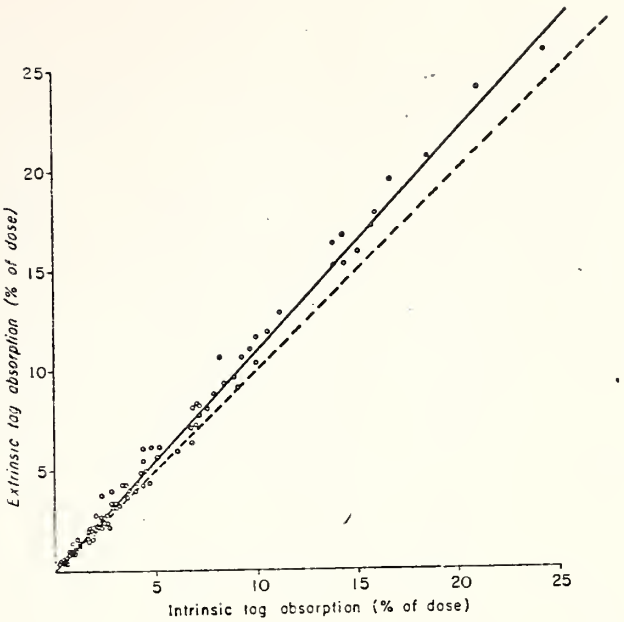

FIGURE 1 Food iron absorption from test meals tagged simultaneously with intrinsic and extrinsic radioiron. Included are all studies performed with an extrinsic tag of 0.1 mg iron or less added to a normal test meal before administration (studies 1, 2, 4, 5, 8, 9, 12, and 14 listed in Table III). The mean ratio between absorption of extrinsic and intrinsic radioiron for the composite data is 1.10 (95% confidence limits 1.09–1.10) calculated as the slope of the least squares regression line fitted through the origin and represented by the solid line. The interrupted line represents a 1:1 ratio.

To permit statistical comparison of the E:I ratio calculated for the various studies listed in Table III, it was necessary that the results have similar variability in the ratio estimate. After excluding studies 6, 7, and 11 which had the highest, and study 9, the lowest values for the SE of the estimate, homogeneous variance was established with the remaining 10 studies. It could then be shown that the ratio of 1.28 observed with separate administration of the extrinsic tag to a complete meal (study 14) was significantly higher, and the ratio of 0.97 observed with an extrinsic dose of 0.5 mg Fe as $FeCl_3$ added to maize (study 3) was significantly lower than the group as a whole. The ratio among the remaining eight studies are plotted in Fig. 1. They showed no statistical differences when tested by covariance analysis ($F = 1.59$, 7, 82 degrees of freedom, $P > 0.10$).

DISCUSSION

The daily turnover of iron in normal human subjects is small, amounting to only about 0.9 mg in adult men

Study	Test meal	Extrinsic tag		No. of Subjects	Age‡	Hematocrit‡	Tsf.‡ sat.§
		Dose	Method*				
		mg Fe			*yr*	%	%
1	Maize	0.001	Mixed A	13	30 ±4	40 ±1	23 ±3
2	Maize	0.1	Mixed A	18	30 ±2	40 ±1	26 ±3
3	Maize	0.5	Mixed A	12	36 ±4	38 ±2	15 ±3
4	Maize	0.001	Mixed B	12	31 ±5	40 ±1	26 ±3
5	Maize	0.1	Mixed B	15	33 ±3	40 ±1	21 ±3
6	Maize	0.1	Separate	11	33 ±5	43 ±1	31 ±3
7	Soybean	0.1	Separate	11	31 ±6	42 ±1	25 ±4
8	Wheat	0.1	Mixed A	13	41 ±4	38 ±1	23 ±3
9	Black bean	0.1	Mixed A	8	33 ±7	41 ±1	28 ±4
10	Maize plus 500 mg desferrioxamine	0.1	Mixed A	12	41 ±2	41 ±1	23 ±2
11	Maize plus 500 mg ascorbic acid	0.1	Mixed A	14	41 ±4	40 ±1	23 ±4
12	Maize plus veal	0.1	Mixed A	8	45 ±5	39 ±2	19 ±4
13	Maize with complete meal	0.1	Separate	11	22 ±1	41 ±1	34 ±4
14	Maize with complete meal	0.1	Mixed A	11	22 ±1	41 ±1	34 ±4

* In methods A and B, the extrinsic tag was added to the intrinsically labeled food, after and before boiling, respectively.
‡ Mean ±SEM.
‖ Geometric mean. Values in parenthesis represent ±1 SEM calculated on the logarithmic scale and retransformed as antilogarithms.
§ Transferrin saturation.
¶ Slope ±SE of the slope for a least squares regression line fitted through the origin.
** SE of the estimate.

(25). The absorptive process plays a key regulatory role, but current methods for measuring food iron absorption are unsatisfactory. Chemical balance studies lack the sensitivity required to measure the small quantity of iron absorbed from the normal diet. Biosynthetic labeling provides accurate measurements of iron availability from individual foods but not from a mixture of foods. The present study was undertaken to determine whether adding an extrinsic tag of inorganic radioiron to a normal meal might provide a reasonable measure of nonheme iron absorption.

There is some evidence in the literature to suggest that an extrinsic tag might be suitable. Sharpe, Peacock, Cooke, and Harris (26) found that marked inhibition of absorption occurs when food is added to a test dose of inorganic radioiron, and suggested that the degree of inhibition is related to the bulk of food added. The addition of an extrinsic tag to a standard meal was later proposed by Pirzio-Biroli and coworkers (27) as a practical approach to studying clinical abnormalities in the absorption of food iron. While the standard meal technique has since been widely applied for this pur-

Iron absorption			Absorption ratios			
			Intrinsic‖	Extrinsic‖	Extrinsic/Intrinsic	
Intrinsic‖	Extrinsic‖	Reference‖	Reference	Reference	b + S_b¶	SEE**
	% of dose					
2.7	3.1	29.8	0.09	0.10	1.10 ±0.01	0.29
(1.9–3.8)	(2.2–4.3)	(23.9–37.2)	(0.06–0.13)	(0.08–0.14)		
4.8	5.0	48.7	0.10	0.10	1.06 ±0.01	0.39
(3.9–5.8)	(4.1–6.1)	(43.3–54.6)	(0.08–0.12)	(0.09–0.12)		
3.9	3.8	49.1	0.08	0.08	0.97 ±0.02	0.30
(3.3–4.6)	(3.2–4.5)	(41.5–58.3)	(0.07–0.09)	(0.07–0.09)		
1.6	2.0	30.0	0.05	0.07	1.12 ±0.03	0.42
(1.2–2.3)	(1.5–2.7)	(23.6–38.2)	(0.04–0.07)	(0.05–0.08)		
4.0	4.6	45.2	0.09	0.10	1.12 ±0.01	0.35
(3.6–5.2)	(3.6–5.9)	(36.3–56.2)	(0.07–0.11)	(0.08–0.13)		
2.6	3.8	18.5	0.14	0.21	1.43 ±0.12	2.99
(1.6–4.2)	(2.3–6.3)	(13.6–25.1)	(0.09–0.23)	(0.13–0.34)		
6.3	7.8	42.2	0.15	0.19	1.27 ±0.06	2.19
(4.3–9.1)	(5.4–11.3)	(30.6–58.2)	(0.10–0.22)	(0.13–0.26)		
2.6	3.0	21.6	0.12	0.14	1.18 ±0.02	0.49
(2.0–3.4)	(2.3–4.0)	(18.1–25.9)	(0.09–0.16)	(0.11–0.18)		
1.5	1.2	25.5	0.06	0.06	0.99 ±0.05	0.26
(1.2–2.0)	(0.8–1.7)	(23.0–28.2)	(0.05–0.07)	(0.03–0.07)		
0.6	0.7	40.7	0.01	0.02	1.14 ±0.04	0.12
(0.5–0.7)	(0.6–0.8)	(35.5–46.7)	(0.01–0.02)	(0.01–0.02)		
22.0	22.5	36.5	0.60	0.62	1.01 ±0.01	1.12
(16.9–28.5)	(17.3–29.2)	(30.1–44.2)	(0.52–0.70)	(0.53–0.71)		
9.7	11.6	47.0	0.21	0.25	1.16 ±0.01	0.45
(7.5–12.5)	(9.2–14.6)	(40.4–54.5)	(0.16–0.26)	(0.20–0.31)		
5.6	7.2	—	—	—	1.28 ±0.02	0.70
(4.4–7.1)	(5.7–9.3)					
3.4	4.0	—	—	—	1.06 ±0.02	0.61
(2.5–4.7)	(2.9–5.3)					

pose (26–33), any implication that the results provide a valid measure of iron absorption from the meal has been carefully avoided.

Schulz and Smith (4) compared the absorption of intrinsic and extrinsic iron, reporting a mean absorption of 9.1% in 10 children given milk labeled in vivo as compared with the mean of 10.6% in 10 children given a test dose of milk to which radioiron had been added. In a similar study with a test meal of eggs, a mean absorption of 11 and 12% with intrinsic and extrinsic tagging was reported in five and three children, respectively. These studies did show a general agreement in the two labeling methods, but they did not supply sufficient data to permit firm conclusions about the general validity of the extrinsic tag method.

In the present study, dual isotope measurements of absorption from a single test dose of food labeled intrinsically with ^{55}Fe and extrinsically with ^{59}Fe, have provided precise estimates of the relative absorption of the two labels. For example, correlation coefficients between log percentage absorption of intrinsic and extrinsic radioiron given in the same test meals were greater

than 0.99 in all but two of the studies listed in Table III (exceptions were studies 6 and 7 in which the extrinsic tag was not mixed with the test meal of single food). The close correlation is also reflected in the SE of the ratio estimate which was less than ±5%. While the 10% greater absorption of the extrinsic tag was statistically significant, this magnitude of error is of little concern in the evaluation of food iron absorption, and appropriate adjustments can be made if necessary.

Several methods of adding the extrinsic tag to the test meal yielded similar results. Thus, no differences were observed between ferrous and ferric salt; nor did varying the dose of the extrinsic tag from 0.001 to 0.1 mg iron have any measurable effect. The finding of a slightly lower ratio of 0.97 when the extrinsic tag dose was 0.5 mg Fe is in keeping with other studies of food iron supplementation in which a ratio close to unity was obtained with much larger doses of extrinsic tag.[2]

Administering the extrinsic tag separately from the test meal of a single food causes a significantly greater variation in the E:I ratio among the individual subjects. This effect was presumably due to incomplete mixing of the tag with the smaller amount of food, because separate administration of the tag with a complete meal gave a variability in the E:I ratio no greater than when the ^{59}Fe was mixed with the food. With smaller test meals, as well as with the complete meal, separate administration of the extrinsic tag was associated with a significantly higher E:I ratio of approximately 1.3. However, in studies with a complete meal, it appears preferable to accept this higher ratio associated with separate administration of the extrinsic tag rather than to homogenize the meal, because the reduced palatability of the homogenized meal may in itself have an adverse effect. This is suggested by the significantly reduced absorption of intrinsic radioiron from homogenized (study 13) vs. unhomogenized (study 14) food. The subjects in this study were able to ingest the homogenized meal only after it had been partially frozen.

A theoretical objection to extrinsic tagging was raised by Moore after a careful review of data obtained by biosynthetic tagging and with the standard meal technique (34). Although in normal subjects the two methods gave comparable results, in iron-deficient subjects the mean absorption of 43.4% in 38 subjects given a standard meal was appreciably higher than the mean of 18.9% in 48 patients given biosynthetically labeled foods. This suggestion that an extrinsic tag cannot be relied upon to measure the absorption of native food iron in subjects with iron deficiency is not supported by the findings in the present study. In applying the criteria established in 1968 by a scientific working committee of the WHO (35) to the 158 subjects listed in the appendix, 16.5% were anemic (hematocrit below 36% in women and 39% in men), 22.8% were iron-deficient based on a serum iron below 50 μg/100 ml and 25.3% were iron-deficient as defined by a transferrin saturation below 15%. A total of 112 or 70.9% of the subjects were normal by all the criteria listed above, although if the level of inorganic iron absorption is taken as a criteria of iron status (36), the mean absorption of 33.1% in the female population and 38.8% in the male subjects suggests a high prevalence of iron depletion. In this mixed population of normal and iron-deficient subjects, no correlation was found between the E:I ratio and the transferrin saturation ($r = -0.08$, $P > 0.2$).

The practical application of extrinsic tagging in studies of food iron absorption must take into account our present understanding of the absorptive mechanism of food iron. There is now good evidence that iron is absorbed in two forms: reduced ionized (nonheme) iron and heme iron. While nonheme iron absorption is altered by the addition of either blocking substances such as desferrioxamine or phytate, or enhancing substances such as ascorbic acid or animal muscle, the absorption of heme iron is unaffected. We have obtained evidence in other studies that with extrinsic tagging, heme iron absorption must be considered an independent system.[2] Total iron absorption from a meal containing both animal and vegetable foods can be measured by determining heme and nonheme iron in the meal by chemical methods, and by employing double extrinsic tags of heme and inorganic iron. However, because heme iron constitutes little, if any of the dietary iron in geographic areas where iron deficiency is most prevalent, and because heme iron absorption appears to be independent of the diet composition, measurement of nonheme iron absorption is perhaps of more immediate concern in studies of food iron availability.

The present finding that absorption of iron added to food closely approaches the absorption of native nonheme food iron has important implications for understanding food iron absorption. The emphasis in absorption studies with isolated test doses of biosynthetically tagged foods has been directed to the biological form of iron within the food. The findings in the present study however, suggest that a common pool of nonheme iron is formed by foods ingested in the same meal, and that the availability of this iron is determined by the composite effect of substances in the meal which either block or facilitate absorption. It would seem appropriate in future studies to clarify the nature of these factors and to define their relative importance in the absorption of dietary iron.

[2] Layrisse, M., and C. Martinez-Torres. Unpublished observations.

APPENDIX—TABLES IV AND V

TABLE IV

Absorption of Food Iron from Test Meals Tagged Simultaneously with Intrinsic and Extrinsic Radioiron

	Sex and age	Hematocrit	Serum iron	Transferrin saturation	Iron absorption			
					Intrinsic	Extrinsic	Reference	Extrinsic / Intrinsic
		%	µg/100 ml	%	%			
Ferric chloride 0.001 mg iron, added to maize after boiling								
1	F 24	36	57	21	0.4	0.6	17.3	1.25
2	F 39	40	80	34	0.4	0.6	23.7	1.25
3	F 48	39	87	27	0.7	0.8	38.0	1.17
4	F 54	41	113	37	1.1	1.6	16.0	1.40
5	F 22	39	48	14	1.7	1.7	14.4	1.07
6	M 25	47	69	18	1.6	1.8	61.6	1.14
7	F 15	44	114	34	4.3	4.9	27.1	1.13
8	M 25	45	107	28	5.1	5.7	43.6	1.11
9	M 32	41	53	17	6.1	6.0	4.8	0.98
10	M 23	41	39	9	7.2	7.8	41.8	1.08
11	M 18	44	69	21	7.8	8.1	40.3	1.04
12	M 15	36	88	21	7.9	8.9	91.1	1.13
13	M 50	32	38	9	18.6	20.7	75.3	1.11
Ferric chloride 0.1 mg iron, added to maize after boiling								
1	F 27	41	72	27	1.7	2.1	26.8	1.27
2	M 48	44	99	29	2.0	2.1	47.8	1.06
3	F 33	43	74	18	2.0	2.8	75.7	1.39
4	M 37	46	126	37	2.1	2.2	42.3	1.05
5	F 26	38	80	29	2.4	2.3	48.2	0.95
6	F 22	39	73	23	2.6	2.8	31.7	1.09
7	M 15	43	182	53	2.7	2.2	17.8	0.83
8	M 45	44	128	36	2.9	3.2	47.8	1.12
9	F 34	35	65	17	3.7	4.0	74.6	1.09
10	F 22	39	92	32	4.4	4.3	35.8	0.98
11	M 35	47	98	30	4.7	4.4	19.2	0.95
12	F 21	41	134	41	6.8	6.4	54.6	0.95
13	F 39	37	62	25	7.0	7.3	51.8	1.05
14	M 18	48	154	49	9.1	9.2	63.8	1.01
15	M 45	35	20	6	13.9	15.3	81.7	1.10
16	M 18	24	12	3	14.4	15.4	81.0	1.07
17	F 35	33	29	6	15.1	16.0	85.9	1.06
18	M 18	41	52	14	24.4	26.0	82.1	1.06
Ferric chloride 0.5 mg iron, added to maize after boiling								
1	F 54	46	93	28	1.6	1.6	19.7	1.00
2	F 46	38	45	14	1.9	1.7	22.3	0.89
3	M 51	38	25	6	2.2	2.1	41.8	0.95
4	M 16	44	66	18	2.6	2.6	98.0	1.00
5	F 47	25	13	3	3.8	3.8	50.2	1.00
6	M 34	44	37	8	3.9	4.0	81.8	1.03
7	F 22	41	112	29	4.4	4.4	46.4	1.00
8	F 25	25	12	2	4.4	4.3	20.0	0.98
9	F 27	37	26	6	4.7	4.8	69.6	1.02
10	M 50	46	126	32	5.7	6.2	64.7	1.10
11	M 16	41	105	30	9.1	8.3	98.2	0.91
12	F 40	28	13	3	9.8	9.3	66.1	0.95

TABLE IV—(*Continued*)

	Sex and age	Hematocrit	Serum iron	Transferrin saturation	Iron absorption			
					Intrinsic	Extrinsic	Reference	Extrinsic / Intrinsic
		%	*µg/100 ml*	%		%		
Ferric chloride 0.001 mg iron, added to maize after boiling								
1	F 30	39	107	35	0.2	0.4	28.9	2.00
2	F 63	40	79	23	0.4	0.6	8.7	1.25
3	F 14	39	93	23	0.6	0.7	31.3	1.20
4	F 40	40	54	16	0.7	1.0	20.1	1.50
5	F 57	39	66	22	1.8	2.0	5.3	1.13
6	F 35	40	110	34	1.9	1.9	22.0	1.00
7	F 30	41	126	33	1.9	2.2	39.7	1.18
8	F 19	42	77	24	2.3	3.8	38.3	1.62
9	F 20	40	65	19	2.6	2.4	46.0	0.96
10	F 39	38	89	28	3.6	3.7	58.1	1.03
11	M 14	40	143	40	6.8	7.2	79.4	1.07
12	F 14	43	38	10	10.6	12.0	89.1	1.14
Ferric chloride 0.1 mg iron, added to maize after boiling								
1	M 50	38	45	12	0.4	0.6	62.8	1.25
2	M 50	43	42	14	1.0	1.0	—	1.00
3	M 55	41	118	32	1.2	1.4	16.3	1.18
4	M 23	45	180	52	1.7	2.0	16.2	1.20
5	F 40	36	50	13	2.3	2.7	—	1.14
6	F 24	38	80	20	2.8	3.4	87.9	1.24
7	F 24	40	47	13	4.1	4.4	63.1	1.08
8	F 45	37	70	19	6.9	8.2	61.6	1.19
9	M 16	36	21	4	7.1	8.4	96.6	1.19
10	F 25	41	100	27	7.2	8.3	68.6	1.15
11	F 17	41	141	37	8.4	9.4	67.8	1.12
12	F 48	42	51	13	9.7	11.1	90.7	1.15
13	M 21	48	112	32	10.0	10.4	39.4	1.04
14	F 33	37	68	19	10.0	11.7	39.7	1.17
15	M 25	32	24	6	15.8	17.3	90.0	1.10
Ferric chloride 0.1 mg iron, administered with maize separately								
1	M 65	40	76	25	0.1	0.1	31.4	1.00
2	M 35	40	97	32	0.4	0.6	2.9	1.25
3	M 38	45	121	30	1.0	1.7	8.9	1.67
4	M 45	46	104	29	1.2	2.8	47.1	2.27
5	M 24	48	147	53	1.7	1.7	16.0	1.00
6	M 36	49	53	15	3.4	7.1	8.1	2.06
7	M 36	44	103	27	7.4	10.0	9.2	1.34
8	M 16	43	112	35	8.4	15.9	30.9	1.88
9	M 15	38	115	33	11.2	22.1	80.7	1.97
10	M 15	40	97	27	12.7	14.3	59.1	1.13
11	F 36	40	105	39	12.9	14.3	13.0	1.11
Ferric chloride 0.1 mg iron, administered with soybean separately								
1	M 78	44	112	36	0.2	0.3	22.2	1.50
2	M 23	43	98	31	3.1	2.9	14.8	0.93
3	M 17	42	136	42	4.2	5.3	5.0	1.26
4	M 21	42	41	13	5.2	7.7	85.1	1.47
5	M 21	47	145	36	9.4	10.1	13.6	1.07
6	M 22	46	78	19	10.1	11.4	67.0	1.13

TABLE IV—(*Continued*)

	Sex and age	Hematocrit	Serum iron	Transferrin saturation	Iron absorption			
					Intrinsic	Extrinsic	Reference	Extrinsic / Intrinsic
		%	*μg/100 ml*	%		%		
Ferric chloride 0.1 mg iron, administered with soybean separately—(*Continued*)								
7	F 16	41	91	20	10.2	14.0	111.4	1.37
8	M 37	34	31	7	10.3	16.8	117.6	1.62
9	F 60	42	131	38	10.4	11.1	91.0	1.06
10	M 34	38	45	9	17.7	19.2	93.6	1.09
11	M 17	44	57	20	21.1	30.1	53.8	1.43
Ferric chloride 0.1 mg iron, added to wheat after boiling								
1	F 23	39	74	24	0.8	0.8	48.2	1.00
2	F 43	43	128	35	0.9	0.9	20.2	1.00
3	F 45	39	88	30	0.9	1.4	13.1	1.63
4	F 55	38	68	22	0.9	1.4	17.1	1.63
5	F 50	37	51	19	1.2	1.2	17.2	1.00
6	F 22	38	83	21	2.2	2.4	13.8	1.10
7	F 20	37	122	39	2.3	2.2	8.6	0.95
8	F 28	42	109	34	3.2	3.3	9.4	1.03
9	F 34	41	85	26	4.0	4.0	21.6	1.00
10	F 60	38	28	7	4.8	6.2	51.9	1.30
11	M 60	35	27	7	8.2	10.7	66.7	1.30
12	F 40	26	14	3	9.3	10.7	35.9	1.14
13	F 56	39	74	20	14.3	16.8	20.3	1.17
Ferric chloride 0.1 mg iron, added to black bean after boiling								
1	M 46	42	108	35	0.5	0.2	18.6	0.36
2	F 22	40	112	43	0.6	0.4	20.8	0.59
3	F 14	39	98	22	1.5	1.2	41.2	0.81
4	F 22	40	78	21	1.7	1.5	23.8	0.90
5	M 56	45	51	20	1.9	1.6	18.4	0.84
6	M 63	43	131	44	2.2	2.2	28.6	0.99
7	F 25	39	44	12	3.0	3.4	25.7	1.12
8	F 14	39	84	21	3.1	3.2	34.6	1.01
Ferric chloride 0.1 mg iron, added after boiling to maize plus 500 mg desferrioxamine								
1	M 44	44	88	25	0.2	0.4	63.7	2.00
2	F 37	35	138	29	0.2	0.2	55.1	1.00
3	F 55	39	106	29	0.3	0.3	36.3	1.00
4	F 41	40	75	32	0.3	0.4	27.2	1.33
5	M 39	45	69	22	0.3	0.6	31.4	1.67
6	M 49	43	78	20	0.7	0.9	69.3	1.33
7	F 46	39	118	34	0.7	0.8	35.7	1.17
8	F 44	38	56	17	0.8	0.9	12.9	1.14
9	F 33	40	65	21	0.9	1.1	40.8	1.25
10	M 32	46	63	13	1.0	1.2	40.1	1.22
11	F 38	38	69	21	1.3	1.4	59.8	1.08
12	M 30	37	19	5	1.4	1.4	60.9	1.00
Ferric chloride 0.1 mg iron, added after boiling to maize plus 500 mg ascorbic acid								
1	F 70	40	99	24	3.4	3.4	8.8	1.00
2	M 55	43	75	30	4.1	4.3	10.5	1.06
3	F 40	38	44	13	8.5	9.0	31.4	1.05
4	F 53	44	48	13	15.6	16.6	20.1	1.06

TABLE IV—(*Continued*)

	Sex and age	Hematocrit	Serum iron	Transferrin saturation	Iron absorption			
					Intrinsic	Extrinsic	Reference	Extrinsic / Intrinsic
		%	*µg/100 ml*	%		%		
Ferric chloride 0.1 mg iron, added after boiling to maize plus 500 mg ascorbic acid—(*Continued*)								
5	M 52	40	150	44	17.7	18.3	69.0	1.04
6	M 51	38	163	54	19.3	19.1	20.6	0.99
7	M 33	48	91	24	20.4	20.2	34.6	0.99
8	M 40	42	83	25	21.7	21.9	49.3	1.01
9	F 40	39	65	17	32.2	33.8	61.4	1.05
10	M 45	38	38	9	48.6	49.7	72.3	1.02
11	F 33	26	14	2	49.3	52.2	69.0	1.06
12	M 17	41	104	35	52.9	52.3	69.0	0.99
13	M 23	37	18	4	67.7	69.7	34.8	1.03
14	M 17	42	73	18	78.3	77.0	70.9	0.98
Ferric chloride 0.1 mg iron, added to maize after boiling and administered with veal								
1	M 56	41	57	17	2.8	4.0	36.8	1.43
2	F 52	37	55	19	4.4	5.5	36.6	1.25
3	F 40	38	33	9	7.2	8.3	38.0	1.15
4	M 52	43	98	29	11.2	12.9	78.4	1.15
5	F 15	40	74	17	13.9	16.4	22.9	1.18
6	M 38	46	122	38	16.0	17.9	71.9	1.12
7	F 58	36	43	13	16.7	19.6	61.8	1.17
8	F 48	28	15	4	21.1	24.2	58.0	1.15

TABLE V

Absorption of Maize Labeled with Intrinsic and Extrinsic Radioiron and Administered with a Complete Meal

	Sex and age	Packed RBC volume	Serum iron	Transferrin saturation	Iron absorption			
					Normal meal		Homogenized meal	
					Intrinsic	Extrinsic	Intrinsic	Extrinsic
		%	*µg/100 ml*	%		%		
1	F 18	39	145	45	2.7	3.7	4.4	6.1
2	F 19	40	151	52	2.8	3.7	5.2	6.2
3	F 21	42	121	28	2.8	3.3	1.2	1.3
4	F 23	42	130	47	3.5	4.3	3.5	4.3
5	F 23	41	116	34	3.5	4.8	0.9	1.0
6	F 34	46	109	34	5.2	6.3	3.4	4.3
7	F 18	34	52	19	8.0	9.8	2.5	2.4
8	F 18	41	116	34	9.7	14.0	4.5	5.0
9	F 20	46	199	39	11.3	15.6	2.4	2.7
10	F 18	41	116	29	13.8	17.7	8.9	9.7
11	F 25	40	32	10	32.0	40.2	38.0	39.9

ACKNOWLEDGMENTS

It is a pleasure to acknowledge the help and suggestions of Dr. Eloise Giblett in preparation of this manuscript for publication.

This investigation was supported by U. S. Public Health Service grants HE-06242, AM-13067, HE-06508, and (Training Grant) AM-5130, by Department of Agriculture Grant 12-14-100-9918 (61), by the Williams-Waterman Research Corporation, and by the World Health Organization. A portion of this work was conducted through the Clinical Research Center facility of the University of Washington supported by NIH grant FR-37.

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