1971) suggests that thiourea may be effective in amounts smaller than indicated in the experiments cited. Fuller (1963) observed that thiourea at a rate of 50 lb of nitrogen per acre gave an increased barley yield over urea and other fertilizers. The considerations discussed are based on mixing thiourea with soil; broadcast application would approximate this, but placement or use in irrigation water would involve less soil per acre and would reduce the amount of thiourea required.

Hauck (1972) has pointed out that one of the requirements for a nitrification inhibitor is that it move with the fertilizer nitrogen in order to maintain an effective concentration throughout the soil-fertilizer reaction zone. The mobility of thiourea in soil has not been established, nor has the effect of the interaction of thiourea and ammonia on mobility been determined, but the polar nature and relatively low solubility of thiourea might be expected to cause it to remain close enough to adsorbed ammonia to preserve its effectiveness as a nitrification inhibitor.

In actual practice the utility of thiourea will depend on the amounts actually required under field conditions and on the cost of thiourea. At a large volume price of 10 cents per pound for thiourea, which appears possible, and at the lower levels indicated to be effective, the cost of using thiourea could approach costs for the more active nitrification inhibitors. The large amounts of thiourea required and its ease of application in solution would make it easy to avoid variations in rate, which would be an important advantage. Use with crops in the field is obviously required to complete the picture on thiourea as a practical nitrification inhibitor.

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Biological Assessment of Available Iron in Food Products

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A variety of fortified and unfortified food products were assayed for "available iron" using rat assays. It seems clear that the "curative assay," based upon the hemoglobin response of animals after prior iron depletion, is more satisfactory than the "prophylactic assay." Iron availability varied greatly and in some products fortified with ferric orthophosphate the added iron appeared to be essentially unavailable. In others, however, ferric orthophosphate appeared to be a satisfactory form of iron. It seems apparent that the availability of iron in a food product may depend upon the form of iron added, the nature of the foodstuff fortified, and possibly the manufacturing process. Measurement of "available iron" by estimating the absorption of a small dose of ^{59}Fe added to the foodstuff did not appear to yield a satisfactory estimate of available iron.

The renewed interest in iron deficiency and the fortification of foods with iron has reemphasized the need for a method to determine the availability of iron in foods. The situation is complex since not only the kind of iron added to foods or the kind of iron in foods, but the nature of the food to which the iron is added and the foods consumed with the iron source influence the availability of the iron. Through the use of radioiron the differences in the availability of iron for human subjects in various sources have been clearly shown (Moore and Dubach, 1951; Hussain *et al.*, 1965; Layrisse *et al.*, 1969; Cook *et al.*, 1969). However, the difficulty of preparing labeled sources which duplicate commercial sources limits the utility of this approach as a routine method. The large intra- and intersubject variability in iron absorption under apparently similar conditions also necessitates the use of relatively large groups of subjects if reasonably accurate estimates of the availability of iron in human subjects are to be obtained.

It is not clearly established how well the iron absorption in any animal species duplicates that in man, but many similarities are evident and it appears necessary to develop satisfactory animal assays for the routine testing of food products. Presumably, future comparative work will elucidate the relevance of such data to human nutrition.

The studies reported in this paper were done to further investigate the use and accuracy of the bioassay procedure previously reported (Amine *et al.*, 1972) for various iron preparations and fortified and unfortified food products. Comparative data were also obtained on the absorption of radioiron added to the diets containing these materials, *i.e.*, the extrinsic labeling of foods which has been pro-

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Table I. Products Tested and Data Obtained in Experiment I

Sample no.	Iron source	Description	Levels of Fe tested, ppm	Body wt,[a] g	Hemoglobin,[a] g/100 ml	Hematocrit,[a] %
	None, basal diet only		0	92.2 ± 28.3	6.8 ± 1.1	24.3 ± 2.6
1	Ferrous sulfate	Standard	5	82.2 ± 8.3	8.9 ± 1.3	28.3 ± 3.9
			10	140.2 ± 12.0	10.3 ± 1.3	33.0 ± 3.2
			15	157.0 ± 11.9	12.7 ± 1.6	38.8 ± 4.6
2	Reduced iron 1	Mean particle size 30.7 μ, 76% of the particles > 20 μ	5	150.6 ± 11.4	8.5 ± 1.3	26.2 ± 2.9
			15	120.3 ± 22.8	8.6 ± 1.3	26.5 ± 3.6
			25	137.2 ± 17.3	8.9 ± 1.3	29.7 ± 3.7
3	Reduced iron 2	Mean particle size 14.7 μ, 74% of the particles < 20 μ	5	145.6 ± 12.7	8.7 ± 0.6	26.8 ± 2.5
			15	141.8 ± 16.2	9.2 ± 1.2	29.0 ± 2.7
			25	146.3 ± 16.6	9.6 ± 1.0	32.3 ± 4.1
4	Reduced iron 3	Mesh analysis 0% on 200; 5% on 325 mesh; particle size not available	5	91.3 ± 16.3	7.9 ± 1.0	25.5 ± 2.9
			15	113.2 ± 10.0	8.6 ± 1.2	28.2 ± 4.9
			25	133.0 ± 11.6	8.9 ± 0.6	30.2 ± 3.2
5	Short bread cookies	Fortified with elemental iron	5	108.2 ± 9.3	7.0 ± 1.4	24.8 ± 3.4
			10	106.0 ± 11.9	8.5 ± 0.8	28.5 ± 1.6
			15	131.0 ± 11.5	9.9 ± 1.0	32.0 ± 1.7
6	Rich salted crackers	Fortified with elemental iron	5	134.0 ± 28.5	6.5 ± 0.6	23.2 ± 1.6
			10	143.6 ± 10.0	8.3 ± 2.2	27.8 ± 4.7
			15	150.3 ± 6.5	10.1 ± 1.6	32.7 ± 2.4
7	Farina 1	Unfortified	10	117.7 ± 15.2	7.2 ± 0.6	24.5 ± 1.4
			20	162.0 ± 15.8	8.5 ± 0.3	28.0 ± 1.4
			30	154.3 ± 18.7	9.3 ± 0.9	31.8 ± 2.9
8	Farina 2	Unfortified, contained baked apple	5	130.7 ± 17.4	8.2 ± 0.8	25.8 ± 2.1
			10	148.5 ± 17.3	9.9 ± 2.4	32.5 ± 5.6
			15	150.5 ± 18.9	10.5 ± 1.3	33.2 ± 3.2
9	Farina 3	Unfortified, contained maple brown sugar	5	146.8 ± 10.8	7.4 ± 1.2	25.5 ± 2.9
			10	142.2 ± 16.6	8.0 ± 0.6	27.3 ± 2.3
			15	147.6 ± 20.2	9.4 ± 1.3	29.7 ± 4.4
10	Short bread cookies	Ferric orthophosphate fortified	10	108.2 ± 9.3	7.3 ± 0.9	26.0 ± 3.2
			20	106.0 ± 11.9	7.4 ± 1.1	26.0 ± 4.0
			30	131.0 ± 11.5	7.4 ± 1.1	26.2 ± 3.9
11	Graham crackers	Ferric orthophosphate fortified	10	126.8 ± 20.7	7.3 ± 1.2	25.7 ± 3.3
			20	144.0 ± 20.2	8.2 ± 0.4	29.5 ± 1.4
			30	153.2 ± 12.8	8.9 ± 0.9	30.3 ± 2.5
12	Rich salted crackers	Ferric orthophosphate fortified	10	113.0 ± 22.3	6.7 ± 1.3	24.5 ± 4.1
			20	119.6 ± 17.3	7.3 ± 1.0	26.0 ± 2.3
			30	104.3 ± 17.7	7.9 ± 0.6	29.0 ± 2.3
13	Farina 4	Ferric orthophosphate fortified similar to sample 7	10	88.8 ± 16.7	7.5 ± 1.0	26.3 ± 2.4
			20	103.0 ± 14.5	8.2 ± 0.5	28.0 ± 2.6
			30	101.8 ± 6.8	8.5 ± 1.0	30.2 ± 3.2
14	Farina 5	Ferric orthophosphate fortified similar to sample 8	10	129.5 ± 11.8	7.1 ± 0.5	24.3 ± 1.5
			20	142.8 ± 19.7	7.2 ± 1.4	24.5 ± 2.4
			30	145.0 ± 19.0	7.3 ± 0.5	24.8 ± 1.6
15	Farina 6	Ferric orthophosphate fortified similar to sample 9	10	93.5 ± 11.7	7.0 ± 0.3	24.5 ± 0.3
			20	155.0 ± 13.4	7.3 ± 1.0	24.7 ± 3.6
			30	150.8 ± 13.6	7.3 ± 1.0	25.2 ± 4.3

[a] Mean ± standard deviation.

posed as a method for evaluating the availability of iron in foods (Cook *et al.*, 1972).

MATERIALS AND METHODS

Weanling female rats of the C.D. strain obtained from the Charles River Breeding Laboratories (Wilmington, Mass.) were used in all experiments and they were housed in individual cages. The purified basal diet used was similar to that previously described (Amine and Hegsted, 1971) except that starch replaced glucose as the source of carbohydrate. In the first experiment 276 rats were divided into 46 groups of 6 animals each which were of similar weight. One group received the basal diet low in iron. Three other groups were used to assay each iron source. These received diets containing sufficient amounts of the iron source under investigation to provide three arbitrarily selected levels of iron. The levels and the iron sources investigated are shown in Table I. The food products replaced equal amounts of starch in the preparation of the diets. The diets and distilled water were provided *ad libitum* and the animals were weighed weekly. After 3 weeks the animals were bled from the tail vein for the determination of hemoglobin and hematocrit.

Whereas in the above described experiment the animals were given the experimental diets on the day after the animals were received in the laboratory, the second experiment involved a prior depletion period. Approximately 300 rats were fed the basal diet low in iron for a 4-week period. At this time they were bled and hemoglobin and hematocrit determined. Only animals with hemoglobin levels between 3 and 5 g/100 ml were utilized. These were divided into 46 groups of 6 animals each with approximately the same mean hemoglobin value. One group continued to receive the basal diet low in iron and as in the previous experiment three groups were used to assay each iron source. These received diets containing three arbitrarily selected iron levels provided by the material under test as shown in Table II. All the animals were bled 10 days later for determination of hemoglobin and hematocrit.

Statistical evaluation of the slope-ratio assay was performed as previously described (Hegsted *et al.*, 1968; Amine *et al.*, 1972). Iron determinations followed the method of Bothwell and Mallett (1955).

After each assay was completed animals were selected with hemoglobin levels between 8 and 10 g/100 ml and ar-

Table II. Products Tested and Data Obtained in Experiment 2

Sample no.	Iron source	Form of iron added	Levels of Fe tested, ppm	Body wt,[a] g	Hemoglobin[a] change, g/100 ml	Hematocrit[a] change, %
	None, basal diet only		0	183.0 ± 26.7	1.5 ± 0.2	3.8 ± 0.3
1	Ferrous sulfate		10	205.0 ± 17.0	4.4 ± 0.8	10.7 ± 1.2
			20	206.0 ± 22.5	7.0 ± 0.9	17.5 ± 1.2
			30	194.7 ± 11.3	9.8 ± 0.6	24.3 ± 1.5
2	Ferric sulfate		10	192.7 ± 19.6	3.2 ± 0.4	7.8 ± 1.0
			25	190.0 ± 7.2	5.8 ± 0.5	14.8 ± 1.2
			40	197.3 ± 18.2	8.1 ± 0.6	21.3 ± 1.8
3	Cocoa powder 1	Ferrous fumarate and reduced iron	10	197.3 ± 21.8	2.8 ± 0.3	6.8 ± 1.0
			25	200.5 ± 7.4	4.4 ± 0.3	11.0 ± 1.1
			40	195.7 ± 13.1	6.6 ± 0.6	16.7 ± 0.8
4	Cocoa powder 2	Ferric orthophosphate	10	209.7 ± 18.2	3.0 ± 0.3	7.3 ± 0.8
			25	211.1 ± 27.1	4.9 ± 0.4	12.1 ± 1.2
			40	204.0 ± 8.5	6.7 ± 1.0	16.8 ± 1.2
5	Commercial infant formula 1	Ferrous sulfate	10	190.0 ± 13.3	3.8 ± 0.7	9.6 ± 1.5
			25	209.0 ± 18.1	7.8 ± 0.7	18.6 ± 0.7
			40	206.3 ± 16.3	10.0 ± 0.8[b]	22.8 ± 4.7[b]
6	Commercial infant formula 2	Ferrous sulfate	10	181.1 ± 19.1	3.5 ± 0.3	9.2 ± 0.8
			25	192.5 ± 31.1	5.8 ± 0.6	15.0 ± 1.3
			40	200.5 ± 17.7	8.1 ± 0.8	20.3 ± 2.3
7	Commercial infant formula 3	Ferrous sulfate	10	186.5 ± 19.0	3.6 ± 0.5	8.0 ± 0.9
			25	200.2 ± 17.3	6.5 ± 0.8	15.2 ± 1.6
			40	191.7 ± 24.3	9.3 ± 0.7	22.0 ± 2.1
8	Ethiopian bread made from teff 1	Unfortified	5	177.0 ± 18.5	2.0 ± 0.5	3.8 ± 1.4
			10	183.4 ± 20.7	2.1 ± 0.5	4.0 ± 1.0
			15	177.2 ± 15.0	2.2 ± 0.2	4.5 ± 1.1
9	Ethiopian bread made from teff 2	Unfortified	5	190.3 ± 12.2	0.9 ± 0.7	2.0 ± 1.3
			10	201.8 ± 11.4	1.3 ± 0.3	2.8 ± 0.8
			15	187.5 ± 9.1	1.3 ± 1.1	3.3 ± 1.0
10	Corn flakes (corn cereal)	Unfortified	10	189.0 ± 13.0	1.2 ± 1.8	2.7 ± 2.2
			20	170.0 ± 19.8	1.4 ± 0.2	3.0 ± 1.0
			30	191.2 ± 17.7	1.3 ± 0.4	3.0 ± 1.7
11	Breakfast cereal (blend of corn, rice, wheat, and oats)	Form of iron not specified	10	193.6 ± 13.2	2.6 ± 0.2	6.8 ± 1.0
			25	196.2 ± 14.7	5.2 ± 0.6	12.2 ± 1.5
			40	202.0 ± 13.0	6.9 ± 0.6	18.3 ± 1.9
12	Bran breakfast cereal	Not specified	10	186.5 ± 13.8	2.7 ± 0.4	8.1 ± 0.7
			25	188.3 ± 18.1	5.2 ± 0.6	13.8 ± 1.8
			40	203.0 ± 21.4	7.3 ± 1.1	20.1 ± 2.7
13	Encapsulated ferrous sulfate 1		10	193.5 ± 27.4	4.2 ± 0.4	10.2 ± 1.2
			20	207.7 ± 16.1	6.7 ± 0.6	17.0 ± 1.4
			30	209.0 ± 13.3	9.5 ± 0.4	23.3 ± 1.3
14	Encapsulated ferrous sulfate 2		10	195.0 ± 19.2	3.1 ± 0.4	7.5 ± 0.8
			20	190.7 ± 13.0	4.7 ± 0.5	11.7 ± 1.2
			30	201.2 ± 22.2	6.2 ± 0.8	16.3 ± 1.8
15	Encapsulated ferrous sulfate 3		10	191.8 ± 19.2	4.3 ± 0.7	11.2 ± 1.0
			20	193.5 ± 11.6	7.0 ± 0.5	17.8 ± 1.2
			30	188.4 ± 8.4	9.9 ± 0.5	24.5 ± 1.6

[a] Mean ± standard deviation. [b] Data not included in the estimate of potency.

ranged in 15 groups of 6 animals each. These received the basal diet low in iron for a few days and were then fasted overnight. They were then given 2 g of diet containing 40 μg of iron provided from each iron source to which was added 0.2 μCi of ^{59}Fe as $FeCl_3$. In a few instances when the food source was low in iron only 20 μg of iron was provided. After approximately 2 hr when practically all of the animals had consumed the food, the animals were counted in a whole body counter to determine the total counts consumed. They were recounted 9 days later and after correction of isotope decay the percentage of the counts consumed which were retained was calculated.

RESULTS

The food products, the levels of iron tested, and the resultant body weights and hemoglobin and hematocrit levels of the animals in experiments 1 and 2 are shown in Tables I and II. Each assay included groups of animals which received graded levels of ferrous sulfate. This gave a maximum response per unit of iron in each assay and is considered to be the standard (*i.e.*, 100% available) to which other iron sources are compared, although this is a relative term and does not imply that all the ferrous sulfate is utilized. Gross inspection of the data reveals rather large differences in the response to different forms of iron.

Figure 1 shows some of the dose-response curves from experiment 1. It is not possible to show all of the data and these were selected for illustrative purposes. The slopes of the lines are proportional to the change in hemoglobin per unit of iron in the diet and large differences in response compared to sample 1 (ferrous sulfate, Table I) are apparent. Some materials, as exemplified by sample 10 (fortified short bread cookies, Table I), demonstrate practically zero availability of the iron they contain. All of the dose-response lines in this experiment are essentially linear. However, it should be noted that the animals which received the diet low in iron throughout had a hemoglobin level of 6.8 ± 1.1 g/100 ml at the end of the experiment. It would be expected that projection of the various dose-response lines to the zero iron intercept would yield a value similar to 6.8 but this is obviously not the case in several instances. Sample 6 (rich salted crackers fortified with elemental iron, Table I), for example, yields an intersection of approximately 4.6, over 2 g/100 ml below the expected value. The statistical evaluation of experiment 1 (Table III) also shows a significant difference between intersec-

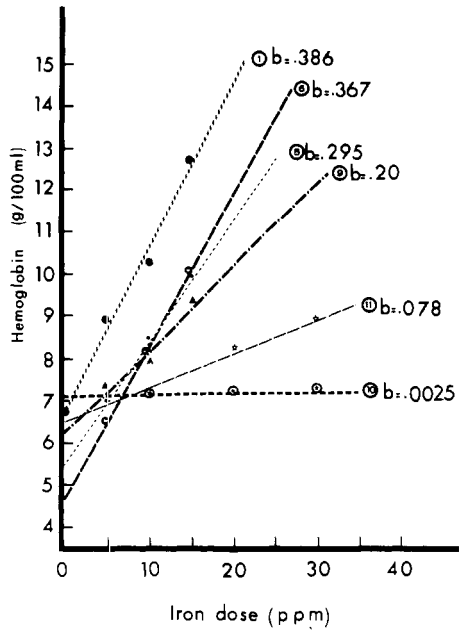

Figure 1. Examples of the dose-response curves obtained in experiment 1. The identifying numbers refer to products in Table I; *b* = slope of curve.

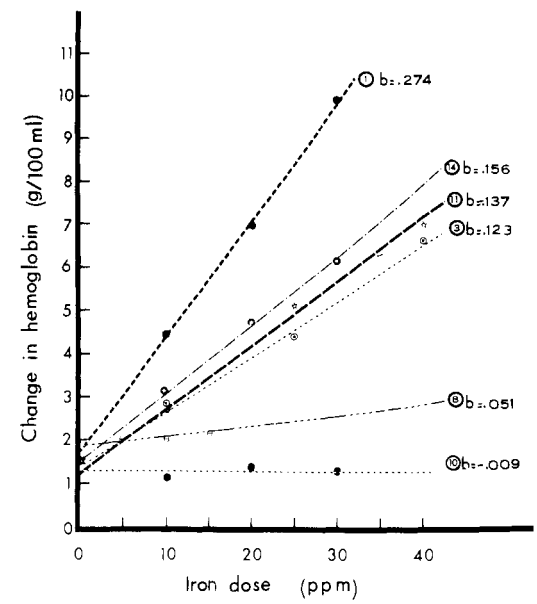

Figure 2. Examples of the dose-response curves obtained in experiment 2. The identifying numbers refer to products in Table II; *b* = slope of curve.

Table III. Analysis of Variance for Experiments 1 and 2 Using Hemoglobin as the Criterion of Response

Source of variation	Sum of squares	Deg of freedom	Mean squares
	Experiment 1		
Due to curvature	4.0819	15	0.27213
Due to intersection	44.3908	14	3.17077[a]
Due to blanks	0.0879	1	0.08790
Due to regression	360.3859	15	24.03158[a]
Error with blanks	338.9340	259	1.30829
Total	699.3199	285	
	Experiment 2		
Due to curvature	5.4747	15	0.36498
Due to intersection	5.9152	14	0.42252
Due to blanks	0.0006	1	0.00062
Due to regression	1936.5177	15	129.10122[a]
Error with blanks	112.1299	259	0.43293
Total	2048.6476	285	

[a] Statistically significant ($P < 0.05$).

tions. The data thus depart to a significant degree from an ideal bioassay in that the intersections are different. The regression line for each sample was therefore calculated individually rather than using the methods appropriate for a satisfactory slope-ratio assay.

Comparable plots for data from experiment 2 are shown in Figure 2. It will be recalled that these animals were depleted of iron prior to the assay and the change in hemoglobin value of each animal rather than the absolute hemoglobin values was used to evaluate the data. The plots reveal large differences in the availability of the iron in the various sources. It should be noted that the animals which received the diet low in iron gained an average of 1.5 g of hemoglobin/100 ml during the assay period undoubtedly because the animals had nearly ceased growing and the basal diet is not free of iron. In this assay, however, projection of the dose-response lines to the zero axis reveals none that depart to a significant degree from the expected value of 1.5. This is verified by the statistical evaluation of the assay (Table III). Differences in the intersections are not significant, the lines do not depart significantly from linearity (curvature is nonsignificant), nor do the intersections depart significantly from the value of the zero dose (blanks). Practically all of the variance is accounted for by regression and this assay has all of the characteristics of an ideal assay.

The estimated potencies relative to ferrous sulfate calculated from the slopes of the regression lines in each assay are shown in Tables IV and V. From these values and the iron content of the product, the amount of available iron can be calculated and is shown in column 6 of each table. For example, sample 5 in Table IV contained 3 mg of iron/100 g and this iron is approximately 76.5% available. Thus, the total available iron is 2.29 mg/100 g. Note that when the same type of cookie is prepared with a relatively high level of ferric orthophosphate (sample 10, Table IV), so it contains 73 mg of iron/100 g, the availability of the total iron was only 0.6% and thus the available iron was only 0.44 mg/100 g—greatly below the value which might be expected.

Farinas 1, 2, and 3 were commercial products specifically said to be unfortified by the manufacturer and Farinas 4, 5, and 6 were the same products specially prepared by the manufacturer with a relatively high level of iron as ferric orthophosphate. Note that the addition of iron salt to Farina 2 to yield Farina 5 was apparently without utility since the total available iron in the original product was the same as that in the fortified product although it contained only 2.95 mg of iron/100 g compared to 45 mg/100 g in the fortified product.

It seems apparent that the availability of the iron in fortified products was generally substantially below that of the natural iron in the products, generally below that of the ferric orthophosphate itself (Pla and Fritz, 1970, 1971; Amine *et al.*, 1972), and that the availability of the iron in the fortified product depended either upon the nature of the product or perhaps on manufacturing procedures. The data also demonstrate, as expected, that sample 2 of reduced iron which had a substantially smaller particle size than samples 1 and 3 was somewhat more available.

Comparable data for the products examined in experiment 2 are shown in Table V. It is of interest that the iron in the two samples of fortified cocoa powder was approximately of equal availability even though one was fortified with a mixture of reduced iron and ferrous fumarate and the other with ferric orthophosphate. Both were much superior to the products containing orthophosphate examined in the previous experiment and the product containing orthophosphate is above that expected from the iron

Table IV. Relative Potency of the Iron in the Various Products Tested in Experiment 1, the Calculated Available Iron, and the Absorption of the Added ^{59}Fe

Sample no.	Iron source[a]	Iron content, mg/100 g	Bioassay			Extrinsic label	
			Slope	Rel potency, %	Available Fe, mg/100 g	^{59}Fe retained, %	Potency rel to ferrous sulfate, %
1	Ferrous sulfate		0.3860	100.0		20.5 ± 2.6	100
2	Reduced iron 1		0.0208	5.4		26.7 ± 4.3	130
3	Reduced iron 2		0.0475	12.2		37.1 ± 3.7	181
4	Reduced iron 3		0.0483	12.5		23.5 ± 3.5	115
5	Short bread cookies	3.00	0.2950	76.5	2.29	30.7 ± 4.6	150
6	Rich salted crackers	3.64	0.3670	94.7	3.45	22.0 ± 4.2	107
7	Farina 1	7.62	0.1080	28.0	2.13	25.0 ± 4.7	122
8	Farina 2	2.95	0.2320	60.0	1.77	33.0 ± 7.8	161
9	Farina 3	3.08	0.2000	51.8	1.60	32.5 ± 5.5	159
10	Short bread cookies	73.40	0.0025	0.6	0.44	25.4 ± 1.9	124
11	Graham crackers	65.30	0.0775	20.0	13.06	18.2 ± 2.0	89
12	Rich salted crackers	80.80	0.0583	15.1	12.20	26.8 ± 3.1	131
13	Farina 4	53.06	0.0500	12.9	6.84	29.7 ± 2.4	145
14	Farina 5	45.10	0.0015	3.9	1.75	33.3 ± 5.0	162
15	Farina 6	52.40	0.0117	3.0	1.57	36.0 ± 6.1	176

[a] See Table I for description.

Table V. Relative Potency of the Iron in the Various Products Tested in Experiment 2, the Calculated Available Iron, and the Absorption of the Added ^{59}Fe

Sample no.	Iron source[a]	Iron content, mg/100 g	Bioassay			Extrinsic label	
			Slope	Rel potency, %	Available Fe, mg/100 g	^{59}Fe retained, %	Potency rel to ferrous sulfate, %
1	Ferrous sulfate		0.274	100.0		17.2 ± 3.7	100
2	Ferric sulfate		0.166	60.5 ± 2.6		10.5 ± 0.7	61
3	Cocoa powder 1	29.9	0.123	44.9 ± 2.4	13.4	32.4 ± 4.1	189
4	Cocoa powder 2	30.9	0.132	48.1 ± 2.4	14.9	25.6 ± 3.9	148
5	Commercial infant formula 1	11.2	0.223	81.5 ± 3.0	9.1	36.8 ± 5.5	214
6	Commercial infant formula 2	12.2	0.167	61.1 ± 2.6	7.5	39.4 ± 2.2	229
7	Commercial infant formula 3	12.8	0.196	71.5 ± 2.8	9.2	35.0 ± 4.7	203
8	Ethiopian bread 1	35.6	0.051	18.6 ± 6.2	6.6	43.4 ± 4.9	252
9	Ethiopian bread 2	36.5	−0.029	−10.6 ± 3.1	0.0	51.3 ± 7.6	298
10	Corn flakes	5.5	−0.009	−3.6 ± 3.1	0.0	37.5 ± 4.3	218
11	Breakfast cereal	35.4	0.137	49.9 ± 2.5	17.7	29.7 ± 5.1	173
12	Bran breakfast cereal	36.2	0.135	49.5 ± 2.4	17.9	33.0 ± 4.5	191
13	Ferrous sulfate 1[b]		0.262	95.7 ± 3.6		30.7 ± 5.2	178
14	Ferrous sulfate 2[b]		0.156	57.7 ± 3.1		37.4 ± 4.5	217
15	Ferrous sulfate 3[b]		0.276	101.0 ± 3.7		21.2 ± 3.5	123

[a] See Table II for description. [b] Encapsulated.

source itself. Samples 5, 6, and 7 (Table V) were commercially available infant foods and the iron availability was generally high. Samples 8 and 9 were Ethiopian bread made from teff and this product is well known to be exceptionally high in iron, much of which presumably represents contamination (Hofvander, 1968). Although the two samples were of similar iron content, the available iron appeared to be substantially different. The second sample (sample 9) actually yielded a negative value as did an unfortified sample of breakfast cereal (sample 10). Although negative values are surprising, certain products may contain sufficient inhibitors of iron absorption to have a net negative value.

The last three samples in the table were samples of encapsulated ferrous sulfate which had been stored in the laboratory for several months. Two of the samples yielded the expected values similar to ferrous sulfate whereas one, sample 14, gave a value similar to ferric sulfate and may have been oxidized during storage.

Also shown in Tables IV and V in the last two columns is the percentage of radioiron retained when the various products were fed with a small dose of $^{59}FeCl$ and the value expressed as a percentage of the retention obtained with ferrous sulfate. It is clear that these values are not proportional to the values obtained in the bioassay. Indeed, in the second experiment there was a significant *negative* correlation ($r = -0.6$) between the potency of the bioassay and the percentage retention of the isotopic iron. The correlation in the first experiment was slightly negative but not statistically significant. When highly unavailable iron sources are tested under these conditions, the "iron pool" in the gut which becomes labeled is small and more of the isotope is absorbed than when the iron is relatively available.

DISCUSSION

As has been previously emphasized (Hegsted *et al.*, 1968; Amine *et al.*, 1972), a satisfactory bioassay should be statistically valid and should provide an estimate of the error in the estimated potency. For this reason "one-point" assays of the type proposed by Pla and Fritz (1970, 1971) are not considered satisfactory. It must also be emphasized that the accuracy or reliability of any estimated potency depends upon the reliability of the standard dose-response curve as well as that of the unknown. Appropriate statistical designs and analyses utilize the variance in both sets of data to calculate the reliability of the estimated potency (Finney, 1964).

Two general types of bioassays are well known—the parallel line assay and the slope-ratio assay. Either is assumed to be valid provided the data can be shown to be appropriate for the assay. In the slope-ratio assay used in these studies, the primary criteria of an adequate assay are that the dose-response regression lines are linear (curvature is not significant) and that these lines have a common intercept which is similar to that obtained for the zero dose or "blank." The analysis as shown in Table III indicates whether significant departure from these expectations is obtained.

A parallel line assay for available iron which appears satisfactory has been demonstrated by Shah and Belonje (1973). An adequate assay of this kind requires that the log dose-response lines be linear and parallel. The merit of the parallel line assay is that the statistical manipulations for its assessment are simpler than those of the slope-ratio assay.

Generally speaking, the dose-response curves in most bioassays are sigmoid when studied over a large range of doses (Finney, 1964) and whether a parallel line or slope-ratio analysis is most appropriate depends upon the range of doses investigated. If doses are selected which are near the upper limits of the response range, the parallel line assay is likely to be appropriate whereas at lower doses the slope-ratio is more likely to fit. Thus, appropriate doses must be selected which fall within the range of the assay selected and this must be verified by an analysis of the data itself.

In the work reported in this paper the parallel line assay cannot be used to assess the potency of the unknowns because the log dose-response lines are neither linear nor parallel in most cases. Presumably this is because the doses selected were relatively low. In theory, at least, similar results should be obtained with either assay if the doses are selected correctly but this remains to be verified by appropriate trials.

In the previous report (Amine *et al.*, 1972) on the bioassay of available iron the procedure used in experiment 1 of this paper appeared to yield satisfactory results. That is, the animals were fed the various experimental diets after receipt of the animals in the laboratory and varying degrees of anemia developed depending upon the dosage and the availability of the dietary iron. This is the so-called "prophylactic" assay as compared to experiment 2 in which the animals were depleted of iron prior to study and a "curvative" assay was used. It is apparent from the results in this paper that the latter assay yielded much more satisfactory results. We believe that when simple iron salts or concentrated sources of iron are assayed either procedure is appropriate. However, when complex materials are assayed which contain materials which may either enhance or inhibit iron absorption and, particularly when they are of relatively low iron content and large amounts must be added to the diet, the rate of iron depletion is markedly affected. The "curative" assay, however, is relatively short and although materials which enhance or inhibit iron absorption affect the potency, they are less likely to cause a serious discrepancy in the assay itself.

The data clearly demonstrate the necessity of assessing iron availability in final products and that the utility of iron added cannot be determined by prior test of the material which is added. Unfortunately, the ferric orthophosphate which was used in the preparation of several of the fortified products in experiment 1 (Tables I and IV) was not available for study. Earlier studies on this material have indicated an average availability of approximately 11% compared to ferrous sulfate (Amine *et al.*, 1972). The reasons for differences in the availability of different samples of this material are unknown. In any event, there was no uniformity in the availability of the iron in products fortified with ferric orthophosphate. In some samples this appeared to be a useless addition, whereas the cocoa powder fortified with the same material (sample 4, Tables II and V) had an availability as high as that of the sample fortified with ferrous fumarate and reduced iron (sample 3, Tables II and V). It is probable that both the nature of the food product and the manufacturing procedure influence the availability of the iron added.

Cook *et al.* (1969) and Layrisse *et al.* (1973) have provided considerable data from human subjects which show that the absorption of an extrinsic tag (radioiron added to food) is similar to that which occurs when the food iron is labeled (an intrinsic tag). Our data do not disprove this but suggest that data obtained with the extrinsic tag should be interpreted with caution. Even if the absorption of intrinsic iron can be accurately estimated with an extrinsic tag which cannot yet be considered to be proven with regard to all types of foods, many foods and diets may contain unavailable iron provided by contamination of direct fortification. It appears unlikely that the absorption of iron from these sources will be appropriately estimated by the extrinsic tag and, thus, such tests are likely to err in yielding optimistic values of the total available iron. It is possible, of course, that the longer transit times and other physiological differences may make the extrinsic tagging more useful in man than it appears to be in the rat.

It is perhaps worth noting that the total absorption of ferrous sulfate labeled with ^{59}Fe in these studies was approximately 20% of that administered. This low value confirms earlier data (Amine and Hegsted, 1971) and is apparently due to the fact that the basal diet was made with starch as the carbohydrate. In the previous studies approximately 40% was absorbed when the dietary carbohydrate was glucose and this was further increased when the diets contained sucrose or lactose. A problem inherent in the development of appropriate bioassays is the definition of the best basal diet and the fact that materials in the basal diet have to be replaced by the material being assayed. This may cause particular problems when the material to be assayed is of relatively low potency.

Layrisse *et al.* (1973) have concluded that in man the absorption of added iron was minimal in subjects consuming a vegetable diet and speculate that iron fortification may only be useful when the basal diet contains sources of animal protein. Whether the casein used to formulate the basal diet in these studies provides a favorable effect on iron absorption is not known. It is apparent, however, that many factors must be considered in the development of satisfactory bioassays and in the application of the results to human nutrition.

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Amino Acid Composition of Cotton Nectar

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Amino nitrogen constitutes an average 0.04% (3.65 μmol/ml) of the extrafloral nectar of cotton (*Gossypium hirsutum* L.). Twenty-four amino acids were isolated from the nectar. Twenty of the amino acids were identified by gas chromatography of their *n*-acetyl *n*-propyl esters and thin-layer chromatography of their dansyl derivatives. Although the amino acid content of cotton nectar fluctuated quantitatively because of environmental conditions, the amino acid constituents were qualitatively consistent. The possibility of cotton nectar as a dietary source of amino acids for nectar-feeding insects is discussed.

The nectar from cotton (*Gossypium hirsutum* L.) has long been known as a source of food for many beneficial predator insects, as well as for parasites and insect pests of cotton (Trelease, 1879; Lukefahr and Rhyne, 1960; Butler, 1968; Butler *et al.*, 1972). Thousands of cotton leafworm (*Alabama argillacea* Hubner) and American cotton bollworm (*Heliothis armigera* Hubner) moths have been observed feeding on the extrafloral nectaries of cotton (Trelease, 1879). The bollworm (*Heliothis zea* Boddie) and the honey bee (*Apis mellifera* L.) have also been reported as nectar feeders on cotton (Butler *et al.*, 1972). Populations of cotton leafworm and cabbage looper (*Trichoplusia ni* Hubner) moths have been found to be seven to ten times higher on cotton with extrafloral nectaries than on nectariless cotton (Lukefahr and Rhyne, 1960).

It has been known since ancient times that nectar is used by anthophilous insects for the energy-providing sugars that it contains. It has been usually assumed that amino acids were obtained elsewhere. Recently, however, Baker and Baker (1973) surveyed nectar of 266 species of flowering plants growing in California and found that nectar contained sufficient concentrations of amino acids for the nutrition of certain insects. The fact that certain insects are known to feed on cotton nectar (Trelease, 1879; Lukefahr and Rhyne, 1960; Butler, 1968; Butler *et al.*, 1972) suggested that cotton nectar should be examined as a potential source of amino acids. Clark and Lukefahr (1956) reported a partial analysis of cotton extrafloral nectar by paper chromatography. No amino acids were detected. Mound (1962) detected two ninhydrin-positive substances in cotton nectar, but amounts or identities were not determined.

This study was conducted to determine the kinds and amounts of amino acids present in cotton nectar and to discuss the implications of nectar as a source of amino acids for insect nutrition.

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EXPERIMENTAL SECTION

Nectar Collection. Initially, separate analyses were made of extrafloral nectar collected from the *Gossypium hirsutum* L. cultivars Stoneville 213, Stoneville 7A, DPL-16, Stoneville 7A Frego, and Acala 1517-70, as well as nectar collected separately from subbracteal nectaries subtending bolls, squares, and flowers. Separate analyses of nectar collected from greenhouse- and field-grown cottons were also compared. No qualitative differences of amino acids were found among these various sources of extrafloral nectar. Therefore, *G. hirsutum* cv. Stoneville 213 was selected for replicate analyses, because it is the predominant cultivar in the immediate area. The nectar was taken directly from the extrafloral nectaries by 20-μl disposable glass micropipets. Analyses were performed immediately after collection. Separate analyses were performed on ten nectar samples collected at various times over a 4-month period.

Isolation. The cotton nectar was acidified, and the amino acids were isolated by chromatography on a miniature ion-exchange column of cationic resin (Harris *et al.*, 1961). The eluted amino acid fraction was divided into two aliquots. One aliquot, to which a volume equivalent to 1 μmol/ml of phenylalanine (internal standard) was added, was used in the conversion of the amino acids to their stable volatile *n*-acetyl *n*-propyl esters. These were separated by gas chromatography (gc) (Graff *et al.*, 1963; Coulter and Hann, 1968). The Perkin-Elmer Model 900 gas chromatograph used was equipped with dual 2 ft × ⅛ in. stainless steel columns packed with Chromosorb G (H.P.), 80–100 mesh, coated with 0.7% PEG (6 *M*)-0.05% TCEPE, with flame ionization detectors and a Perkin-Elmer Model 56 recorder. The carrier gas (helium) flow was 60 ml/min, the injector temperature was 230°, the detector temperature was 260°, and the column temperature was programmed from 100 to 250° at 12°/min.

The second aliquot was used in the conversion of the amino acids to their stable dansyl derivatives, according to the method of Airhart *et al.* (1973). The dansylated amino acids were then separated by two-dimensional thin-layer chromatography (tlc) on Cheng-Chin polyamide sheets, according to the method of Woods and Wang (1967).