Biological Estimation of Available Iron Using Chicks or Rats

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A slope–ratio assay for the estimation of the biological availability of dietary iron sources using young rats or chicks has been described. Comparison of hemoglobin, hematocrit, and body weight as the measure of response indicates, as expected, that hemoglobin and hematocrit are equally satisfactory, whereas body weight is an insensitive criterion of response. In view of the ease with which the hematocrit can be determined, it is presumably the measure of choice. The data demonstrate that commercial preparations used as iron sources in animal or human foods vary widely in availability. Modest but statistically significant differences in availability are found, depending upon the animals used in the assay, rats or chicks, and whether the animals are made anemic prior to the assay or not. The merit of an adequately designed bioassay procedure which provides not only an estimate of availability of the iron source but also an estimate of the precision of the assay is stressed.

Anemia due to iron deficiency is a commonly recognized problem throughout the world, and the fortification of various foods with iron has long been recommended and practiced as a means of controlling iron deficiency. It now appears, however, that the fortification programs are relatively ineffective (Elwood, 1965). It would seem quite clear that either the sources of iron which are added are inappropriate or that the levels added to fortified food are inadequate.

Differences in the availability of iron in various foods or iron salts have long been recognized (Blumberg and Arnold, 1947). A knowledge of the total iron content of foods or diets is clearly insufficient to evaluate the adequacy of the diet with regard to iron. No useful purpose is achieved and the public is misled if the iron added to foods is not assimilated or varies greatly in the degree to which it can be assimilated. Thus, methods are needed to estimate the amount of "available iron" that foods or diets provide. The estimation of the availability of iron with human subjects is difficult and most effectively done with isotopically labeled iron sources (Hussain *et al.*, 1965). This requirement, together with other problems inherent in working with human subjects, limits the general applicability of this kind of testing procedure. Chemical methods (Henley *et al.*, 1956) of assessing iron availability are still of questionable utility and must, in any event, be validated by biological tests. Thus, there is a clear need for appropriate biological assays. Considerable effort was made in this direction many years ago at about the time when iron fortification was begun (Elvehjem and Kemmerer, 1933; Smith and Otis, 1937; Andrews *et al.*, 1939; Freeman and Burrill, 1945), but little attention has been given to the problem in recent years. Fritz and his collaborators (1970) have revived efforts in this field and have proposed an assay method using either rats or chicks (Pla and Fritz, 1970). This paper reports the results obtained in applying a standard "slope–ratio" assay in either rats or chicks.

MATERIALS AND METHODS

The study consisted of four experiments in which either weanling female rats or 1-day-old cockerels were used. In the first experiment, 114 weanling female Charles River albino rats were divided according to their body weight into 19 comparable groups of six rats each. Eighteen rats were used for each salt tested. Each group of six received a diet containing one of three arbitrarily selected levels of iron for 3 weeks. One group received the basal diet low in iron for the same period of time (Amine and Hegsted, 1971a). Three groups received reagent grade ferrous sulfate ($FeSO_4 \cdot 7H_2O$) containing 20% iron, which served as the reference standard iron salt assumed to be maximally utilized. The other 15 groups were utilized to study iron absorption from four different iron salts and hemoglobin. Salt 1 was a mixture of two lots of ferric orthophosphate prepared for use in cereal enrichment containing 28.6% iron. Salt 2 was a mixture of two lots of sodium iron pyrophosphate prepared for use in cereal enrichment containing 14.5% iron. Salt 3 was a mixture of three lots of reduced iron; one is used for cereal enrichment and the other two are laboratory reagents. The iron content was 97.0%. Salt 4 was a mixture of three lots of ferrous carbonate containing 38% iron used in animal food fortification. The fifth source was hemoglobin iron obtained from Fisher Scientific Co. (Medford, Mass.). Each of these sources was added to the diet at three different arbitrarily selected levels based upon estimates of their probable nutritive value. All salts were mixed with glucose to provide 1 mg of iron/g of mixture and this was added to the basal diet by replacing equivalent amounts of glucose in the basal diet. The rats were individually housed in stainless steel cages and were fed their respective diets *ad libitum*.

After 3 experimental weeks, blood samples were obtained in the rats by amputating the tip of the tail for the determination of hemoglobin (Crosby *et al.*, 1954) and packed cell volume by a microcapillary method. The body weights of the animals were also recorded.

In the second experiment, 100 1-day-old cockerels were fed a diet low in iron (Amine and Hegsted, 1971a) supplying less than 7 ppm of iron for 3 weeks. A random sample of the chicks were bled every week to determine their hemoglobin and hematocrit. At the end of the third week when the chicks had developed severe anemia, all the chicks were bled from the wing vein to determine their hemoglobin and hematocrit. They were then divided into ten comparable groups according to their hemoglobin, hematocrit, and body weight. Each group was housed in a separate galvanized cage with wire-mesh floor. Food and iron-free water were supplied *ad libitum*. One group was continued on the basal diet low in iron. The other nine groups were utilized to estimate iron availability from three iron salts—the standard, ferrous sulfate, ferric orthophosphate, and sodium iron pyrophosphate, as in the rat experiment. All salts were mixed in the diet at three arbitrarily selected levels. At the end of the second week of repletion, hemoglobin and hematocrit were determined again.

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In the third experiment, 130 1-day-old cockerels were randomly divided into 13 groups of 10 chicks each. One group was fed the basal diet low in iron; the other 12 groups were utilized to estimate iron availability from four different iron sources. These included the standard, ferrous sulfate, reduced iron, and ferrous carbonate previously described and an artificial rice containing 0.8% ferric orthophosphate ($FePO_4 \cdot 4H_2O$) prepared for use in rice fortification. After 3 experimental weeks, all the chicks were bled for hemoglobin and hematocrit determinations.

Experiment 4 was similar in methodology to that of Experiment 3. Iron availability was determined for the same iron salts used in the first experiment. It should be noted that in Experiment 2 the chicks were made anemic by feeding all of the animals the same diet for 3 weeks and iron was provided for 2 weeks. In the other experiments the animals were started on the test diets immediately.

Data were also obtained on the absorption of radioiron when trace doses of $^{59}FeCl_3$ were mixed with diets containing the various iron sources. Iron-deficient rats were fasted overnight and then given 2 g of the basal diet containing either 20 or 80 μg of iron plus 0.2 μCi of $^{59}FeCl_3$. The retained iron was determined as the difference between the total body count 2 hr and 9 days after the ingestion of the labeled dose and after correction for the physical decay of the isotope, as in earlier studies (Amine and Hegsted, 1971b).

Statistical analysis for all the experiments was done using the technique previously described for the estimation of protein quality utilizing the slope–ratio assay (Hegsted and Worcester, 1966; Hegsted *et al.*, 1968).

RESULTS

The nature of satisfactory bioassay procedures and the statistical procedures which should be applied to evaluate them have been discussed in detail by Finney (1964). A satisfactory assay yields not only an estimate of the potency of the test substance relative to the standard but an estimate of the variability, *i.e.*, precision, in the estimated potency as well. The standard error of the estimated potency is largely dependent upon the variance of the points around both the dose-response line of the standard preparation and that of the unknown. Finney describes the appropriate statistical procedure.

It should also be noted that in a satisfactory assay the estimate of the potency cannot be dependent upon the dose which happens to be chosen. If this happens, a variety of potencies can be obtained and one does not know what is the true value of the potency. Only by testing the unknown at more than one dose can one check that the potency is independent of dosage level.

As will be apparent from the results presented in this paper (Tables I and II), the slope–ratio assay (Hegsted and Worcester, 1966; Hegsted *et al.*, 1968), using either the hemoglobin levels or hematocrit levels in chicks or rats as the measure of response, appears to be quite satisfactory. The data from Experiment 4 (Table II) are typical of those obtained in these studies. Figure 1 shows the dose-response regression lines calculated for each of the iron salts independently. The slopes, "b," are indicated for each line. Note that values for two groups (see Table II) were omitted in the calculations, since the dose was apparently too high to lie on the linear portion of the curve. The comparable lines and slope obtained from the computer program (Hegsted *et al.*, 1968) which forces the lines through a common intercept are shown

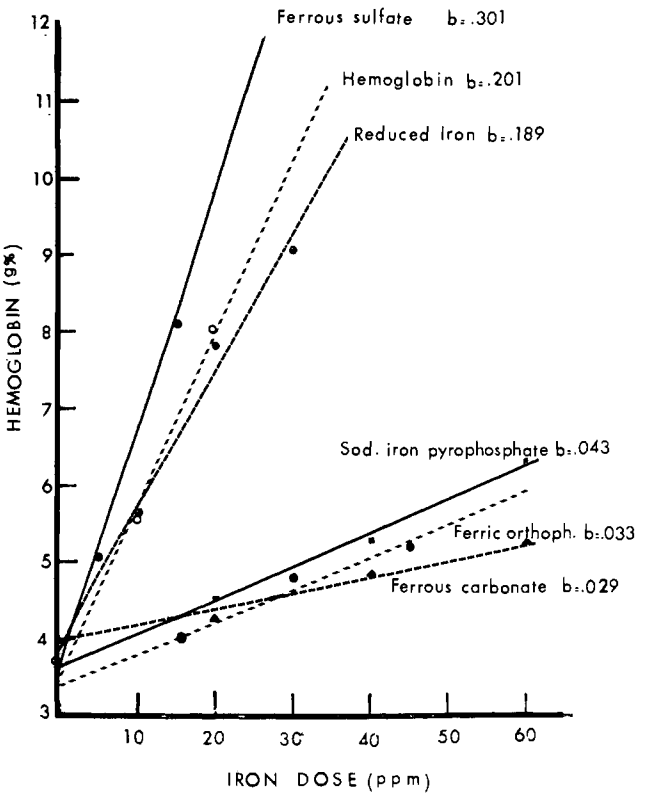

Figure 1. Dose-response regression lines and slopes calculated independently for each of the iron salts tested in Experiment 4

Table I. Mean Terminal Values and Standard Deviations for Hemoglobin, Hematocrit, and Body Weight of Rats Fed Different Levels of Iron Sources in Experiment 1

Iron source	Iron dose, ppm	Hemoglobin, g/100 ml	Hematocrit, %	Body weight, g
Basal diet	0	5.3 ± 1.1	21.9 ± 3.5	103 ± 21
Ferrous sulfate	10	8.8 ± 0.7	32.0 ± 1.4	130 ± 7
Ferrous sulfate	20	11.2 ± 1.4	39.7 ± 4.1	133 ± 9
Ferrous sulfate	40[a]	13.3 ± 0.8	43.5 ± 3.5	129 ± 7
Ferric orthophosphate	20	7.0 ± 0.7	28.3 ± 1.5	117 ± 14
Ferric orthophosphate	40	8.7 ± 0.7	32.0 ± 2.5	133 ± 11
Ferric orthophosphate	60	10.2 ± 2.0	36.2 ± 4.3	135 ± 17
Sodium iron pyrophosphate	20	5.5 ± 0.8	22.3 ± 2.7	118 ± 6
Sodium iron pyrophosphate	40	5.8 ± 0.6	24.3 ± 1.9	113 ± 12
Sodium iron pyrophosphate	60	6.2 ± 1.0	25.9 ± 2.7	116 ± 10
Reduced iron	10	6.8 ± 0.9	26.2 ± 1.5	113 ± 10
Reduced iron	20	8.2 ± 0.8	31.6 ± 3.6	121 ± 8
Reduced iron	40[a]	9.6 ± 0.5	35.7 ± 1.4	138 ± 14
Ferrous carbonate	20	5.2 ± 0.4	21.8 ± 1.4	109 ± 6
Ferrous carbonate	40	5.3 ± 0.6	22.0 ± 2.0	116 ± 12
Ferrous carbonate	60	5.9 ± 0.9	22.5 ± 3.1	119 ± 14
Hemoglobin	15	7.1 ± 0.9	28.5 ± 2.2	122 ± 7
Hemoglobin	30	8.6 ± 0.8	32.4 ± 1.6	135 ± 18
Hemoglobin	45	10.1 ± 0.5	38.3 ± 2.4	138 ± 12

[a] Data not included in the estimates of potency.

Table II. Mean Terminal Values and Standard Deviations for Hemoglobin, Hematocrit, and Body Weight of Chicks in Experiments 2, 3, and 4

Exp	Iron source	Iron dose, ppm	Hemoglobin, g/100 ml	Hematocrit, %	Body weight, g
2	Basal diet	0	4.0 ± 0.4	16.8 ± 1.8	291 ± 99
	Ferrous sulfate	10	5.8 ± 0.5	22.3 ± 2.5	361 ± 59
	Ferrous sulfate	20	6.6 ± 0.5	24.3 ± 1.2	407 ± 75
	Ferrous sulfate	40[a]	8.8 ± 0.5	29.7 ± 1.4	406 ± 62
	Ferric orthophosphate	10	4.5 ± 0.6	18.8 ± 2.1	314 ± 62
	Ferric orthophosphate	20	4.6 ± 0.4	19.5 ± 1.6	299 ± 118
	Ferric orthophosphate	40	5.0 ± 0.9	20.0 ± 2.1	285 ± 83
	Sodium iron pyrophosphate	10	4.2 ± 0.9	18.4 ± 2.4	270 ± 91
	Sodium iron pyrophosphate	20	5.1 ± 0.7	20.0 ± 1.6	346 ± 98
	Sodium iron pyrophosphate	40	5.7 ± 1.2	22.2 ± 3.8	406 ± 80
3	Basal diet	0	3.6 ± 0.9	16.7 ± 3.8	167 ± 29
	Ferrous sulfate	5	4.7 ± 0.8	18.6 ± 2.3	201 ± 55
	Ferrous sulfate	15	6.6 ± 1.1	23.7 ± 2.6	299 ± 53
	Ferrous sulfate	30	9.0 ± 0.4	29.0 ± 0.9	254 ± 69
	Reduced iron	10	5.3 ± 0.5	20.5 ± 1.6	210 ± 43
	Reduced iron	20	6.5 ± 0.3	23.3 ± 1.0	227 ± 45
	Reduced iron	40	8.4 ± 0.7	27.6 ± 1.9	225 ± 75
	Ferrous carbonate	20	3.5 ± 0.5	17.0 ± 2.1	154 ± 34
	Ferrous carbonate	40	4.0 ± 0.5	17.9 ± 1.3	181 ± 34
	Ferrous carbonate	60	4.8 ± 0.5	18.0 ± 2.4	158 ± 35
	Rice fortification mixture	10	4.1 ± 0.8	18.8 ± 1.9	177 ± 38
	Rice fortification mixture	20	4.7 ± 0.7	21.3 ± 3.1	203 ± 45
	Rice fortification mixture	40	6.6 ± 1.4	25.1 ± 3.7	215 ± 53
4	Basal diet	0	3.7 ± 0.9	17.1 ± 1.9	187 ± 40
	Ferrous sulfate	5	5.1 ± 0.9	20.8 ± 1.3	250 ± 38
	Ferrous sulfate	15	8.2 ± 0.9	28.8 ± 2.6	282 ± 43
	Ferrous sulfate	25[a]	9.3 ± 0.5	31.0 ± 1.5	250 ± 33
	Ferric orthophosphate	15	3.9 ± 0.5	18.0 ± 2.9	206 ± 57
	Ferric orthophosphate	30	4.8 ± 0.6	19.0 ± 2.9	220 ± 53
	Ferric orthophosphate	45	5.2 ± 0.9	20.0 ± 2.7	212 ± 48
	Sodium iron pyrophosphate	20	4.5 ± 0.3	19.1 ± 1.8	198 ± 35
	Sodium iron pyrophosphate	40	5.4 ± 0.4	20.5 ± 1.9	219 ± 55
	Sodium iron pyrophosphate	60	6.3 ± 0.6	23.2 ± 1.7	248 ± 38
	Reduced iron	10	5.6 ± 0.9	20.5 ± 2.6	238 ± 46
	Reduced iron	20	7.8 ± 0.8	25.0 ± 2.2	250 ± 54
	Reduced iron	30	9.1 ± 0.5	28.2 ± 1.9	242 ± 38
	Ferrous carbonate	20	4.3 ± 0.9	17.7 ± 3.3	175 ± 42
	Ferrous carbonate	40	4.9 ± 0.9	19.0 ± 2.6	159 ± 21
	Ferrous carbonate	60	5.4 ± 0.6	19.6 ± 2.1	229 ± 64
	Hemoglobin	10	5.6 ± 0.8	23.1 ± 1.6	208 ± 47
	Hemoglobin	20	8.0 ± 0.9	28.2 ± 2.8	236 ± 48
	Hemoglobin	30[a]	8.4 ± 0.8	30.0 ± 1.8	248 ± 44

[a] Data not included in the estimates of potency.

in Figure 2. The similarity of the slopes in the two figures is apparent.

Ferrous sulfate gave the highest slope of the materials tested, showing an increase of 0.304 g of hemoglobin/100 ml for each ppm of iron in the diet. This value is assumed to represent a maximum response. The availability of the other sources relative to ferrous sulfate is estimated by comparing the slope of the dose-response line relative to that of ferrous sulfate. Thus, reduced iron with a slope of 0.191 has an estimated potency of 0.191/0.304 × 100 or 63%. Mean terminal hemoglobin and hematocrit values and body weights of the animals in the various experiments are shown in Tables I and II. Table III presents the estimated potency of the various iron sources as determined in the four experiments using hemoglobin as the criterion of response. In the analysis of the data, the values from a few groups which received the highest levels of the iron salts tested were omitted from the analysis. It was apparent from an inspection of the data that there was a linear relationship between dose and response at the lower doses, but that these points departed from the expected line to

Table III. Estimate of the Relative Availability and Standard Errors for the Iron Sources Tested Based on Hemoglobin Levels

Exp	Iron source	Regression coefficient	Slope–ratio	Standard error	95% Confidence limits[a]	
					Lower	Upper
1	Ferrous sulfate	0.299	1.000	...	...	...
	Ferric orthophosphate	0.080	0.268	0.023	0.223	0.314
	Sodium iron pyrophosphate	0.012	0.039	0.024	−0.008	0.085
	Reduced iron	0.146	0.488	0.068	0.355	0.621
	Ferrous carbonate	0.004	0.014	0.024	−0.034	0.062
	Hemoglobin	0.106	0.353	0.031	0.294	0.413
2	Ferrous sulfate	0.115	1.000	...	...	...
	Ferric orthophosphate	0.018	0.153	0.063	0.029	0.276
	Sodium iron pyrophosphate	0.034	0.300	0.056	0.191	0.409
3	Ferrous sulfate	0.203	1.000	...	...	...
	Reduced iron	0.130	0.641	0.058	0.526	0.755
	Ferrous carbonate	0.017	0.081	0.023	0.036	0.127
	Rice fortification mixture	0.073	0.360	0.039	0.283	0.436
4	Ferrous sulfate	0.304	1.000	...	...	...
	Ferric orthophosphate	0.034	0.112	0.017	0.078	0.146
	Sodium iron pyrophosphate	0.043	0.143	0.013	0.117	0.168
	Reduced iron	0.191	0.629	0.036	0.558	0.699
	Ferrous carbonate	0.030	0.097	0.013	0.072	0.123
	Hemoglobin	0.212	0.699	0.047	0.606	0.791

[a] t = 1.960.

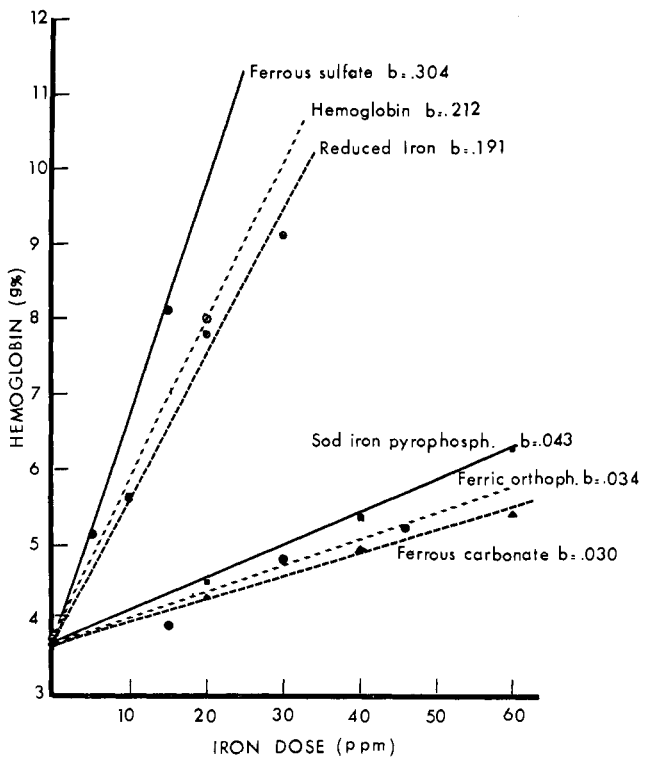

Figure 2. Dose-response lines and slopes for the same data shown in Figure 1 when the lines are forced through a common intercept

a substantial degree. It is obvious that in assays of this kind the linearity of the response as a function of dose must fail if excessive doses are tested and such points should not be considered in evaluating the data. However, as discussed later, it is not entirely clear why the dose-response lines of the specific iron preparations begin to deviate from linearity at different levels of response.

Table IV. Analysis of Variance from Typical Assay When Hemoglobin Is Used as the Response Criterion in Experiment 1

Source of variation	Sum of squares	Degrees of freedom	Mean squares	F
Due to curvature	0.3775	6	0.0629	0.0744
Due to intersection	2.5277	5	0.5055	0.5979
Due to blanks	0.0002	1	0.0002	0.0002
Due to regression	348.1789	6	58.0298	68.6337
Errors with blanks	79.4819	94	0.8455	
Total	427.6608	101		

Complete analysis of variance for the first experiment is shown in Table IV. The assay is quite satisfactory. There is no indication of significant curvature. This indicates that we have similar degrees of utilization at different dosages. Also, there is no significant departure from either the intersections (a fundamental requirement for statistical validity) or blanks.

Table V shows the estimated potency from the iron sources when hemoglobin, hematocrit, or body weight are used as the criterion for iron availability by the rat. Comparable data on the retention of ^{59}Fe when tracer doses were administered with the various iron sources are also presented. The data on hemoglobin iron absorption were available from a previous study (Amine and Hegsted, 1971b). As might be expected, hemoglobin and hematocrit gave essentially the same estimate of potency, whereas body weight gave inconsistently different estimates of potency and is judged an unsatisfactory measure of response. When the radioactive tracer technique was used, the retention of radioactivity had little or no relationship to the expected potency.

DISCUSSION

The material presented demonstrates that the assay procedure for iron availability in either chicks or rats using hemoglobin or hematocrit as the measure of response is technically

Table V. Comparison of the Relative Potency and Standard Errors of Iron Sources Based on Hemoglobin, Hematocrit, and Body Weight on Inorganic Iron Label Retention

Iron source	Relative potency			^{59}Fe retained, %	Potency relative to ferrous sulfate, %
	Hemoglobin, %	Hematocrit, %	Body weight, %		
Ferrous sulfate	100	100	100	59.5 ± 2.3	100.0
Ferric orthophosphate	26.8 ± 2.3	26.7 ± 2.2	33.8 ± 8.4	33.5 ± 2.3	56.3
Sodium iron pyrophosphate	3.9 ± 2.4	5.4 ± 2.2	5.3 ± 7.7	39.6 ± 3.0	66.5
Reduced iron	48.8 ± 6.8	49.6 ± 6.6	27.5 ± 23.2	54.4 ± 2.8	91.4
Ferrous carbonate	1.4 ± 2.4	0.4 ± 2.4	6.9 ± 7.6	46.3 ± 2.7	77.8
Hemoglobin	35.3 ± 3.1	39.6 ± 3.1	52.8 ± 12.5	38.0 ± 3.9[a]	63.9
				22.4 ± 2.2[b]	37.6

[a] Taken from Amine and Hegsted (1971a). [b] Biologically labeled hemoglobin. Taken from Amine and Hegsted (1971a).

satisfactory. Since the estimated potency and the standard errors were essentially the same whether hemoglobin or hematocrit was used as the response criterion, and since hematocrit determinations are much simpler and less expensive than hemoglobin determinations, it is cheaper to use hematocrit in routine assays. Changes in body weight are not a satisfactory measure of iron utilization, since depression of body weight occurs only when the animals are severely anemic.

A comparison of the data obtained in Experiment 1 with rats and Experiment 4 with chicks shows that there is similarity in relative order of availability of different iron salts in the two species. Ferrous carbonate was the least well utilized relative to ferrous sulfate, for example, and reduced iron was the best utilized relative to ferrous sulfate. However, in the rat, ferric orthophosphate was approximately twice as potent as in the chick, and all other iron sources were better utilized in the chick than in the rat. These differences are all statistically significant and require further investigation.

The reproducibility of the assay under comparable conditions (compare Experiments 3 and 4) appears to be very satisfactory even though, as in many bioassays, the confidence limits are occasionally wide.

In Experiment 2 the chicks were made anemic prior to the assay, whereas in the other experiments the animals were started immediately upon the assay diets. The latter procedure has the advantage of shortening the time required for the assay and the animals are bled only once. It is therefore considerably cheaper and faster.

The data available indicate that comparable degrees of precision are obtained by either procedure. However, the potencies of both ferric orthophosphate and sodium iron pyrophosphate were higher when chicks that had been made anemic before being fed the iron salts were used (Experiment 2), the latter significantly so, than in Experiment 4. This may be of some practical significance. It can be argued that the potency for the prevention of anemia is of more significance than the potency for the cure of anemia. This also requires further study and comparison.

It may be noted in Table III that the standard errors are quite variable. Reduced iron, for example, consistently showed a rather large standard error in all of the three assays in which it was tested. This might indicate considerable variability in the ability of individual animals to utilize this salt or it may simply reflect greater variability in hemoglobin levels in animals as the hemoglobin approaches normal values. There is some indication that the materials with relatively higher potencies, reduced iron and hemoglobin, for example, show relatively large standard errors.

The rice fortification mixture was prepared with ferric orthophosphate, yet this showed considerably greater potency than ferric orthophosphate itself when tested in Experiments 2 and 4, which were also the chick assays. It should be emphasized that the materials tested are only samples of these substances and not necessarily representative of those commercially available. The extent to which various samples of any iron salt might vary in availability as measured under these conditions is unknown. It is possible also that the procedures used in the preparation of the rice fortification mixture or other materials contained in the mixture might modify the availability of the iron salt it contains.

The addition of tracer doses of radioiron to diets containing other sources of iron is an unsatisfactory procedure for estimating iron utilization. The total iron in the gut is clearly not uniformly labeled. Presumably the various iron sources are absorbed not only at different rates but possibly at different levels in the gut. The extent to which the label mixes with the "available iron pool" thus appears to be highly variable and unpredictable. It may be noted that when biologically labeled hemoglobin was used, the retention of ^{59}Fe was similar to the estimated availability of hemoglobin iron utilizing the bioassay.

It should be emphasized that the utilization of radioiron sources for measuring iron availability in either man or animals assumes that the labeled iron source exactly duplicates the properties of the usual iron source. Whether this is entirely feasible is open to some doubt. Another serious problem in all bioassays, whether in man or animals, involves the dietary conditions under which the test is made. Since the remainder of the diet (Layrisse *et al.*, 1968) as well as the iron source affects the utilization, the general applicability of data obtained under standardized conditions can be questioned also. Interactions between dietary constituents and various sources of iron have been studied only to a limited extent.

As was previously mentioned and as indicated in Tables I and II, some of the highest doses of the iron preparations tested were omitted from analysis since it was clear that the response departed significantly from the linear relationship between dose and response. Since the maximum response is limited by homeostatic levels of hemoglobin or hematocrit, all such dose-response lines must depart from linearity if the dose tested is too large, and the elimination of data of this kind from analysis is an accepted procedure. Maximum efficiency in an assay will be obtained if all data that do not show significant curvature are included.

An inspection of the dose-response curves for reduced iron in Table I and for hemoglobin in Table II where the highest levels were eliminated may suggest that the level of hemoglobin at which curvature becomes evident may not be solely dependent upon the amount of iron available to the animal,

since in the same assays the region of linear response with other preparations apparently exceeded the level of hemoglobin achieved with these two preparations. There is the possibility that as the animal approaches normality and the utilization of iron begins to fall there may be fairly complex interactions between utilization by the host and the types of iron in the diet. Thus, in this critical area, which would be most important in the prevention of iron deficiency, the relative utilization of iron preparations might be different from those observed with more deficient animals. The data available, however, do no more than suggest that this possibility is worthy of consideration and may explain differences in results obtained with preventive programs as compared to the efficiency of materials in the treatment of anemia.

As has been emphasized, the primary merit of a well designed bioassay procedure involving dose-response curves of both the standard and the unknowns is that the data obtained allow one to determine whether the assay is valid and also the precision of the estimated potency. Such data cannot be obtained if only a single dose of the unknown is tested, as has been recommended (Pla and Fritz, 1970). The five iron salts we have tested were the same samples utilized in the collaborative assays (Pla and Fritz, 1971). As would be expected, the results we are reporting show a general similarity to those found in the collaborative assay. The mean values and the range of values reported from different laboratories were as follows when compared to ferrous sulfate:ferric orthophosphate, 12% (0–33); sodium iron pyrophosphate, 13% (2–29); reduced iron, 46% (27–100); ferrous carbonate, 3% (0–14). These values should be compared or contrasted to the values shown in Table IV and the 95% confidence limits shown. In view of the wide range of values reported from different laboratories for the same material, it would clearly be advantageous to utilize techniques which provide an estimate of the precision of the value reported.

A more important question, of course, is whether the data on iron availability obtained with experimental animals is applicable to man. This cannot be answered until sufficient quantitative data for various species have been accumulated. The data so far available for man show large and unexplained differences between individuals, which makes quantitation difficult. Furthermore, the availability of iron in man, as in other species, depends upon the combination of foods which are fed (Layrisse *et al.*, 1968). Thus, an adequate exploration of this field will require the testing of a large variety of iron sources in combination with an undetermined number of food mixtures. This is a formidable task with human subjects and the use of human subjects for routine testing or development work would appear to be impossible. Less expensive, more accurate and more rapid assays are required.

Animal assays should also define the dietary factors which appear to be of primary importance which may then be selectively studied in man to determine whether they have comparable importance. Since rats or chicks may prove to be inappropriate models, either in general or for particular problems, additional comparative studies with other species should be continued.

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