The Measurement of Iron Absorption

By J. D. Cook, M. Layrisse and C. A. Finch

RECOGNITION that the control of iron balance in normal man resides with the absorptive rather than the excretory process has lead to a heavy investment in studies of iron assimilation during the past few decades. The use of radioisotopes of iron was first described by Hahn and co-workers1 in 1943 and has been followed by the development of highly accurate technics for measuring iron absorption. One of the most convenient of these is the determination of red cell activity after oral administration of radioiron. The assumption that blood activity reflects accurately body activity seems justified in the normal and iron deficient subject when the usual ratios are 80 ± 10 per cent and 90 ± 10 per cent, respectively. In pathologic states with decreased red cell utilization of radioiron, it is necessary to employ a double isotope method, total body counting or stool collections.2 Despite such precise measurements, absorption studies have been difficult because of large differences in absorption among normal subjects and in the same subject with repeated testing.3,4 At the same time the constancy of body iron in a given population suggests that the ingress of iron is not haphazard but is rather a highly regulated process. If absorption is to be further understood, it is necessary to account for the variability observed. In examining iron absorption, two types of studies may be distinguished depending on whether the absorptive machinery of the individual or the nature of the substance ingested is being examined. These two categories are concerned with intersubject and intrasubject differences, respectively.

Intersubject Measurements

The largest variable in iron absorption is the difference between individuals. Measurements with labeled iron salts in apparently healthy subjects show a range from less than 1 to over 50 per cent. This is due to the behavior of the intestinal mucosa and undoubtedly reflects the individual iron balance or requirements of the subject tested.5 Differences in iron balance of the entire population may be found in separate geographic areas, presumably reflecting the type and level of dietary iron supply. Thus in reports from England of mean absorption from a dose of 5 mg. of ferrous iron given to 10 or more subjects, Callender et al.,5 Bannerman et al.6 and Smith and Pannacciulli7 report

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absorptions of 25, 30 and 20 per cent, respectively, while in the United States Brown et al.\(^8\) and Turnbull et al.\(^9\) report values of 7 and 12 per cent. The greater iron requirements in women due to menstrual loss and the demands of pregnancy result in lower iron stores and a proportionately higher assimilation of iron than in men.\(^3\) Studies by Brise and Hallberg\(^4\) show the effects of blood loss on absorption. Two hundred thirty-eight blood donors were found to absorb an average (geometric mean) of 15.7 per cent from a 30 mg. dose of ferrous iron as compared to 5.9 per cent in a nondonor group of 102 subjects. If iron stores are artificially altered, iron absorption is modified in a reciprocal direction over long periods of time until the original equilibrium is established.\(^1\) These findings indicate the importance of selecting individuals with comparable levels of storage iron and evaluating iron absorption in different groups of subjects. However, even if attention is paid to history of blood loss, sex differences, or levels of plasma iron and transferrin, marked differences among select individuals are still observed. There is no good method other than the measurement of iron absorption itself to determine the mucosal setting for iron absorption in the individual subject, and the limitation imposed by subject variation can only be compensated for by employing sufficient numbers of subjects to attain statistical validity.

Analysis of iron absorption data has been previously performed by classic statistical methods which require that the data be reasonably close to normal distribution. In order to determine whether iron absorption measurements can be fitted to a normal distribution, two sets of data were chosen from the literature. In the first series, measurements of absorption were performed in 320 healthy Swedish volunteers by Brise and Hallberg.\(^4\) The test dose contained 30 mg. of ferrous\(^{59}\) sulfate with 0.1 M ascorbic acid/mole of iron and was administered with 4 Gm. of sucrose and 75 ml. of water on each of 5 alternate mornings. Absorption was determined from radioactivity incorporated into circulating red cells 14 days after the final test dose. Blood volume was estimated from body weight and 100 per cent red cell incorporation of absorbed iron was assumed. The second series consisted of 234 determinations in subjects from the outlying area of Caracas, Venezuela. A single test dose of 3 to 5 mg. of ferrous\(^{59}\) sulfate mixed with 2 M of ascorbic acid/mole of iron was administered with a measured volume of water. Absorption was determined as in the first series. Mean absorption in the first series was 16 per cent and in the second, 32 per cent. This difference can be accounted for by the different amounts of iron administered and by the incidence of iron deficiency anemia of 30 per cent in the South American series whereas iron deficiency anemia was specifically excluded in the Swedish study. Despite these differences in mean absorption, the frequency distribution curve in both series is highly skewed in a positive direction and bears little resemblance to the theoretical normal curve\(^8\) (Fig. 1).

The hypothesis of normality of the frequency distribution was evaluated with the Chi Square Test for Goodness of Fit.\(^1\) Highly significant Chi Square values of 101.5 and 85.1 were obtained for the first and second series, respectively, (22 d.f.) indicating wide departure from a normal distribution.
MEASUREMENT OF IRON ABSORPTION

Fig. 1.—Frequency distribution of iron absorption expressed as per cent of the administered dose. The series represented in the upper figure consists of 320 measurements of healthy, Swedish subjects. The lower figure shows the frequency distribution of 234 determinations in subjects from Caracas, Venezuela. In each, the solid line represents the observed frequencies while the shaded area depicts the theoretical normal distribution as determined by the mean and standard deviation. The enhanced absorption in the Venezuelan group is the result of a lower iron dose as well as a higher incidence of iron deficiency among subjects tested.

The approach most commonly employed for evaluating data with a skewed distribution involves transforming the units of measurement to a scale which normalizes the frequency distribution. Both the square root and logarithmic transformation were applied to the two series of data and only log transformation was found suitable for both as shown in Figure 2. Through this manipulation, the sensitivity of methods of statistical analysis is improved.

Another requirement of classic statistical tests is that the variance (square of standard deviation) remains constant at different levels of mean absorption. This assumption is particularly important for the two-sample t test which has been extensively applied to iron absorption. In a total of 38 series of measurements, each containing at least 12 individual measurements, the stan-

*The Chi Square values for testing the goodness of fit of the transformed measurements to a normal distribution were as follows: for the first and second series, respectively, values for the square root transformation were 52.3 and 20.5 (18 and 18 d.f.) and for the logarithmic 27.2 and 38.1 (14 and 20 d.f.).
standard deviation was found to be not constant but rather increased linearly as mean absorption increased (Fig. 3a). For all series in which the individual observations were published, the relationship between the mean and standard deviation were again examined after transforming percentage absorption to the log scale and were then found to be independent of the mean (Fig. 3b).

An alternate statistical approach of equal validity to that of logarithmic transformation is the use of nonparametric methods in which no assumption about frequency distribution is required. The most important group are rank order statistics in which observations are arranged in order of magnitude and where the difference between adjacent observations is made uniform by then using their rank value. When either distribution-free technics or the logarithmic transformation approach were employed in evaluating iron absorption measurements, no difference in sensitivity was apparent, but both were more sensitive than the customary approach.

While the precision of clinical studies may be improved by applying these more sensitive methods of analyses, it is important to recognize limitations still imposed by intrasubject variations. As an example, let us assume a population similar to the group reported by Brise and Hallberg. A certain group of sub-

Fig. 2.—Frequency distribution of iron absorption data after logarithmic transformation of the data shown in the corresponding graphs of Figure 1. The solid line and shaded areas represent the observed and theoretical normal distributions, respectively.
Fig. 3.—Relationship between the mean and standard deviation in several reported series of radioiron absorption

In the upper figure (a) mean absorption is expressed as per cent of the administered dose. The close linear relationship between the mean and standard deviation invalidates statistical methods which assume homogeneous variances and reflect a highly skewed distribution of the measurements. The lower figure (b) represents the relationship between mean and standard deviation when individual values for per cent absorption were first transformed to their logarithms. By this maneuver the requirements of a standard deviation independent of mean has been satisfied.

jects suspected of having an abnormal iron absorption is to be compared to a control group. Estimates of the combined number of test and control subjects required to give an 80 per cent chance of demonstrating a significant difference \((P = 0.05)\) in mean absorption are shown in Table 1. It is evident that a large number of subjects are required, and that the number is greater when an increase in absorption is to be identified.

INTRASUBJECT VARIATION

A major category of absorption studies is that designed to evaluate the relative availability of different forms of administered iron. The objective in experimental design is to minimize subject differences in order to evaluate
Table 1.—Absorption Study Design

<table>
<thead>
<tr>
<th>Difference in mean absorption (%)</th>
<th>Number of subjects required *</th>
<th>(-)</th>
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<tr>
<td>30</td>
<td>156</td>
<td>280</td>
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<tr>
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<td>80</td>
<td>11</td>
<td>59</td>
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* The estimates of group size (representing both the control and test subjects) are based on an 80 per cent chance of demonstrating a difference at the 5 per cent level with the formula

\[
\frac{T - C}{C} \times 100
\]

where \( T \) and \( C \) represent mean percentage absorption of iron in the test and control groups, respectively; \((-)\) represents a decrease in absorption by test subjects as compared to controls and \((+)\), an increase.

the effect on iron absorption of the form in which the material was administered. When only two forms of iron are to be tested, these may be compared in the same subject. However, it is usually desirable to establish absorption in absolute terms rather than as a comparison of two subjects. This can be accomplished by comparing all test substances to some reference standard, such as ferrous ascorbate. It has been possible to express the absorption of a type of food iron in a hypothetical individual who has an absorption of ferrous iron at any specified level. The most suitable type of iron compound to be employed as a reference standard is probably a ferrous iron salt, since this represents the final common pathway by which all forms of dietary iron with the exception of hemoglobin are assimilated. Hemoglobin iron has some advantage if it is more independent of gastrointestinal secretion and motility but has the disadvantages of a unique pathway of absorption possibly at a different level of the intestinal tract, and a narrower range of absorption.

In comparison between iron compounds particular attention must be directed to intrasubject variations in absorption. While the mucosal response can be standardized by comparing the absorption of food iron against a common reference standard, day to day variations in the same subject are still appreciable. This physiologic variation from two doses given on successive days accounts for a range from −50 per cent to +80 per cent \((\pm 1 \text{ S.D.})^*\) of mean absorption assigned the value of 100 per cent. Methodologic error plays

*In order to express the variability in iron absorption measurements as the S.D., the latter has been calculated from the logarithms of percentage iron absorption. Since the logarithmic S.D. is unfamiliar, the limits of the mean ± 1 S.D. have been retransformed as antilogarithms to recover the original units of percentage absorption. The asymmetrical confidence limits derived in this manner provide a more realistic description of variability for the skewed frequency distribution of iron absorption. For example, when repeated measurements are made within the same subject with an overall mean absorption of 10 per cent, roughly two-thirds of the values representing the limits of ±1 S.D. would fall within 50–180 per cent of the mean or 5–18 per cent absorption.
Fig. 4.—A comparison of the absorption of two types of food iron in two groups of subjects whose absorption has also been measured by ferrous ascorbate. The percent absorption of iron in each food was nearly identical as shown on the left. However, the simultaneous measurement of ferrous Fe$^{55}$ ascorbate absorption in these subjects showed a significant difference in absorption by the two groups. The ratio was, therefore, expressed between the absorption of food iron and iron salts as shown on the right. In order to evaluate the significance of this difference it was necessary to express these ratios as logarithms. The difference in these ratios as evaluated by the Student t-test is highly significant ($p < 0.001$), and shows the greater availability of iron in veal as compared with fish.

little part in this since two doses of iron given at the same time show a variation of $\pm 2$ per cent. In studies reported by Brise and Hallberg the physiologic variation has been reduced to approximately $-10$ to $+25$ per cent of mean absorption by administration of five test doses of each form of radioiron alternately over a 10 day period. The advantage of multiple dose administration will depend on the type of iron compound being evaluated. For inorganic forms which can be easily prepared and taken by the subject, multiple dose methods seem most appropriate. When more elaborate preparation of test material is required as in studies of food iron absorption, as much or more information may be obtained by administration of single doses to more subjects.

An important aspect of the dual tracer technic for studying absorption of dietary iron is analysis of data. For each pair of observations, the absorption index is calculated from the ratio of the test dose absorption compared to the absorption of ferrous ascorbate in the same subject. Since these values have a distribution similar to those shown in Figure 1, it is necessary to convert them to logarithms in order to apply statistical analyses. The log value is used to establish a mean absorption index which then permits comparison of absorption between various types of food. Estimates together with confidence limits
obtained on logarithmic scale are retransformed in order to express results in original units. To demonstrate the use of this model a comparison of absorption from two different types of dietary iron in two groups of subjects having a different basal absorption is outlined in Figure 4. This comparison illustrates the importance of a common reference standard in evaluation of iron availability in different foods.

SUMMARY

Sources of variation in iron absorption measurements and methods of controlling them have been reviewed. There are marked differences in the mean level of absorption in different individuals and appreciable day-to-day variation in absorption in the same individual. Intersubject variations can be reduced by the selection of individuals with a more uniform iron requirement, but large variations remain which can be dealt with only by applying appropriate statistical methods to the skewed data obtained and by studying sufficient subjects to provide statistical validity to the results. Comparative studies of the availability of food iron are best carried out against a reference standard in the same subject, and intrasubject variations can be reduced by multiple dose administration.

REFERENCES

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