# Modification of Iron Toxicity and/or Absorption in Mice

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**A study was made of the toxicity of iron administered orally in mice under various conditions. In a quantitative 6-group technique α-aminoacetic acid effected a statistically significant increase in mortality rate of iron. Conversely, sodium succinate reduced the mortality rate of iron with statistical significance. Comparisons of slopes of dosage response curves of mortality rates of iron administered orally with and without additives showed very close parallelism.**

IRON COMPOUNDS have received considerable attention regarding methods of improving absorption and decreasing irritation. Dietary factors have been found to have a great influence upon the absorption of iron. Gastric acidity, ascorbic acid, SH groups of proteins, and other reductants in foods help convert ferric iron to the apparently more readily absorbable ferrous form.

Many different iron compounds alone or with other additive compounds have been employed in the attempt to increase iron absorption and decrease the usual side effects of orally administered iron. Iron choline citrate chelate (1, 2), ferrous fumarate (3–5), ferrous aminoacetosulfate (6–10), an iron carbohydrate complex (11), and combinations of iron with polyol monolaurate (12), polysorbate 20,[1] polysorbate 80[2] (13, 14), magnesium aluminum hydroxide (15), and D-sorbitol (16–18) are several common examples.

It was, therefore, the purpose of this study to demonstrate any influence which glycine and sodium succinate had on the mortality rate of orally administered iron, a greater mortality resulting from increased toxicity or absorption and a reduced mortality resulting from decreased toxicity or absorption at a given dose of iron.

## EXPERIMENTAL

In the initial stages of this toxicity study it was observed that unfasted animals gave erratic results when fed iron compounds orally (19). Likewise, it was found that mice fasted 22 ± 2 hr. were much more uniformly sensitive to iron toxicity than were unfasted mice. Also, although mice fasted under conditions preventing their exposure to feces and sawdust have been shown to increase their susceptibility to iron toxicity (19), the Fairfield Webster female mice in this study were all fasted 22 ± 2 hr. with water *ad libitum*, and exposed to both of these elements to prevent the complication of cannibalism. The animals weighed approximately 25 Gm. The room temperature of 24° was kept as constant as possible. All medication was given by the oral route *via* stomach intubation. The doses of all compounds were calculated on a milligram per kilogram basis and the number of animals dead after 24 hr. was observed; the remaining animals were observed for a period of 30 days for any possible latent effects.

To limit variability, the following experimental design was employed in which 60 mice were divided at random into the following six groups for concurrent testing.

**Group A**—Controls; received 0.3 ml. distilled water.

**Group B**—Additive; received an additive compound without iron (at one or more subsequent dose levels).

**Group C**—Iron standard; a solution containing 125 mg. of ferrous sulfate (25 mg. of iron) per ml.,[3] referred to hereafter as ferrous sulfate solution, dose 160 mg. Fe/Kg.

**Group D**—Iron standard; ferrous sulfate solution, 240 mg. Fe/Kg.

**Group E**—Ferrous sulfate solution, 240 mg. Fe/Kg. and additive at dosage level of group *B*.

**Group F**—Ferrous sulfate solution, 240 mg. Fe/Kg., and additive at dosage level of group *B*.

All six groups received the solutions within a 1-hr. period. The experiment was repeated to give totals of 20 mice per group. The iron doses were chosen so as to provide a range for observation of possible protection as well as potentiation of the iron toxicity by the additive compounds.

The additives were employed as 20% solutions. Two doses of glycine were employed in the attempt to determine the effect of the additive on the toxicity of iron.

The data from the six-group design were analyzed to determine statistically significant differences between the iron administration alone and with the additive. The basis for the estimation of toxicity was a quantal response in which a probit analysis was employed [Finney (20), Burns (21), Fisher and Yates (22)]. The analyses, per cent mortality values, $\rho$ values, and confidence limits were calculated on each individual group of ten animals as well as on the data from the combined groups giving 20 animals per group so as to observe any day-to-day variation, if present.

For confirmation of the analyses of the additives found to be significant, five 27-Gm. mice were fasted 22 ± 2 hr. with water *ad libitum*, and given orally 3.2 mg./Gm. α-aminoacetic acid and 48 mcg. Fe/Gm. Another group of five 27-Gm. mice were similarly fasted and given orally 3.2 mg./Gm. sodium succinate and 48 mcg. Fe/Gm. Both groups were allowed 1 hr. before sacrificing and subsequent separation of the gastrointestinal tract from the remainder of the carcass. The five carcasses and five gastrointestinal tracts of each group were combined and digested with sulfuric and nitric acids until only a clear solution in sulfuric acid with

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[1] Marketed as Tween 20 by Atlas Chemical Industries, Inc., Wilmington, Del.

[2] Marketed as Tween 80 by Atlas Chemical Industries, Inc., Wilmington, Del.

[3] Marketed as Fer-In-Sol by Mead Johnson and Co., Evansville, Ind.

a small amount of white precipitate remained. These four resulting solutions were cooled, made up to 100 ml. in a volumetric flask, and used as earlier described under the modified Fisher method for the determination of iron used in this laboratory (23). The colorimetric analysis was made after the iron was liberated and oxidized with sulfuric acid and potassium persulfate and treated with potassium thiocyanate.

Calculations of slopes of dosage response curves for iron administered orally with and without additives were performed to determine their parallelism.

## RESULTS AND DISCUSSION

The data obtained using the six-group design are presented in Table I.

The results of the statistical analyses of the separate and combined data are summarized as follows. Glycine, 3.2 mg./Gm., potentiated the toxicity of orally administered iron and sodium succinate, 3.2 mg./Gm., decreased the toxicity of orally administered iron. Both of these results were shown to represent statistically significant differences.

The results of the chemical determinations of iron content in mice in which the additive agents produced statistical results are given in Table II.

The dose of 48 mcg. Fe/Gm. mouse for a 27-Gm. animal would amount to 1.296 mg. iron and added to the previously published average iron content per mouse of 1.457 mg. would be a calculated total of 2.753 mg. or 102 mcg. Fe/Gm., compared to the determined average figure of 2.270 mg. and 84 mcg. Fe/Gm. An average of 483 mcg. Fe or 18 mcg. Fe/Gm. mouse was unaccounted for, as noted previously, probably due to the inherent properties of the colorimetric method itself.

However, the averages of iron content in the respective glycine and succinate gut, 840 and 1000 mcg., represent iron absorption of 456 and 296 mg., respectively, on the basis of the calculated 1.296 mg. of iron administered orally. This serves to support the statistical significance arrived at in the statistical calculations, *i.e.*, glycine potentiates while sodium succinate inhibits iron absorption.

In these experiments, calculations of slopes of dosage response curves in which mortality rates of iron administered orally without additives were obtained. The mean slope was 7.038, compared with a mean slope of 7.577 for the average of groups in which iron and additives were administered together. This very close parallelism between slopes would appear to indicate that death was due to iron toxicity and that the effect of additives was merely to modify absorption.

The 60 mice in the control groups receiving oral intubations of distilled water were without deleterious effects and were alive after the 30-day observational period, indicating that the technique itself was not a contributing factor in this toxicity study.

## SUMMARY AND CONCLUSIONS

A study was made of the absorption of iron administered orally to mice under various conditions.

Of the additive compounds tested using the quantitative 6-group method, glycine showed a statistically significant increase in the mortality of iron. Likewise, sodium succinate gave a statistically significant reduction in the mortality of iron. These significant results were verified by oral administration of the compounds with iron, subsequent digestion, and chemical determination of the iron content by the modified Fisher method reported earlier.

Calculation of slopes of dosage response curves, in which mortality rates of iron administered orally with and without additives were compared, showed close parallelism.

The technique of oral intubation was demmonstrated to be adequate for the study of absorption and safe, as exemplified by the lack of deleterious effects on the control groups of animals receiving only distilled water.

TABLE I—MODIFYING EFFECT OF ADDITIVES ON THE TOXICITY OF IRON ORALLY IN MICE USING THE STATISTICALLY DESIGNED SIX GROUP TECHNIQUE (PER CENT MORTALITY IN 24 hr.)[a]

| Additive | Group B | Group C | Group E | Group D | Group F |
|---|---|---|---|---|---|
| | | 32 mcg. Fe/Gm. | | 48 mcg. Fe/Gm. | |
| | Additive Alone | Alone | Plus Additive | Alone | Plus Additive |
| Glycine | | | | | |
| 1.6 mg./Gm. | 0 | 10 | 30 | 50 | 60 |
| 3.2 mg./Gm. | 0 | 5 | 15 | 20 | 55 |
| Sodium succinate | | | | | |
| 3.2 mg./Gm. | 0 | 5 | 0 | 40 | 10 |

[a] Sixty mice in group *A* were given distilled water alone and all lived; therefore, group *A* is not included.

TABLE II—AVERAGES FROM MICE IN GROUPS OF FIVE OF IRON PRESENT PER MOUSE CARCASS AND GUT, SEPARATE AND COMBINED

| Group | Av. Iron Content in Carcass, mg. | Av. Iron Content in Gut, mcg. | Av. Iron Content/ Mouse, mg. | Av. Iron Content/ Gm. Mouse, mcg. |
|---|---|---|---|---|
| 3.2 mg./Gm. glycine and 48 mcg. Fe/Gm. | 1.480 | 840 | 2.320 | 86 |
| 3.2 mg./Gm. Na succinate and 48 mcg. Fe/Gm. | 1.220 | 1000 | 2.220 | 82 |

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# Vitamin C and Choline Content of *Chlorella vulgaris* and *C. pyrenoidosa*

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**The choline content of *Chlorella vulgaris* and of *C. pyrenoidosa* (expressed as micrograms per milligram dry cell weight) remained relatively constant during a 3-week growth period. However, the concentration in the latter species exceeded slightly that of the former at all harvest times. In both species, after reaching a peak early in the culture period (5–7 days), the concentration of ascorbic acid decreased continuously. The total yield of both vitamins (micrograms per milliliter of culture) increased continuously because of the increment in cell mass.**

THIS NOTE records data concerning yields of vitamin C (total ascorbic acid) and of choline from two species of unicellular green algae and is part of a continuing study of production of vitamins and accessory growth factors by *Chlorella vulgaris* and *C. pyrenoidosa* (1–5).

## METHODS

The pedigree of the strains of *C. vulgaris* and of *C. pyrenoidosa* employed, the method of maintaining stock cultures, the composition and inoculation of stock and experimental culture solutions, and the details of environmental conditions provided and of harvesting experimental cultures have been described previously (1). In summary, an inorganic medium consisting of minerals and micro-elements was inoculated with the algae and was continuously aerated with a $CO_2$–air mixture. Continuous illumination (600 f.c.) was provided from a Mazda source and the temperature was maintained at 20.5 ± 0.5°. Cells were harvested at intervals during the 3-week culture period and vitamin C and choline content were determined.

**Assays**—*Vitamin C Activity*—Since ascorbic and dehydroascorbic acids exhibit equal antiscorbutic activity, the method of Roe and Osterling (6) was applicable and was employed to determine "total ascorbic acid." This entailed extraction of cells with a metaphosphoric acid mixture and treatment with a decolorizing agent[1] which oxidized ascorbic to dehydroascorbic acid and clarified the solution. The oxidized form was then coupled with 2,4-dinitrophenylhydrazine and the resulting derivative, when treated with $H_2SO_4$, produced a red color which could be quantitated photometrically.

Aliquots of each cell suspension were extracted in duplicate and each extract was assayed in duplicate for total ascorbic acid. The standard deviations of the assays were within ±5% of the mean; most were within ±2%.

*Choline*—Cells were autoclaved in 3% $H_2SO_4$ at 15 lb. pressure for 2 hr. and then were neutralized with BaOH, following the method of Horowitz and Beadle (7). After filtration, the extract was passed through a Permutit column to separate choline from methionine and to eliminate other substances which might interfere with the assay.

Choline content of the extract was determined using *Neurospora crassa* ATCC 9277 as the test organism in bacto-choline assay medium (8). Growth response of the organism was determined gravimetrically.

Aliquots of each cell suspension were extracted in duplicate and each extract was then assayed in duplicate. For all assays the standard deviation was within the range ±10% of the mean, generally falling in the range ±5% or less.

## RESULTS

The maximum ascorbic acid content detected in the cells of both organisms was about 1.1 to 1.2 mcg./mg. of cell dry weight. This peak occurred in the early period of growth (5 to 7 days) and then the concentration declined continuously throughout the remainder of the 21-day culture period (Table I, A). Choline appeared more constant in concentration. The apparent trend in the average values for choline in *C. vulgaris* is not supported by the data from individual runs. Variations were random. Neither compound was detectable in the harvested culture medium.

When results are expressed in terms of micrograms per milliliter of culture, both compounds are seen to have increased continuously throughout the culture period (Table I, B). This is a reflection of the continuous increase in cell mass as growth progressed.

The levels of choline and the peak levels of ascorbic acid (Table I, A) compare favorably with data reported by Morimura (9) for short-term (2–3 days) synchronized cultures of *C. ellipsoidea*. He also found that the choline content remained fairly constant, but reported that ascorbic acid "increased considerably during the stages . . . in which the photosynthetic process occurred most actively." Confirmatory evidence for this view is provided by the facts that (*a*) in the present

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[1] Marketed as Norit A by American Norit Co., Inc., Jacksonville, Fla.