# Binding of Iron to Amino Acids and Serum Proteins *in Vitro*[1] (36021)

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Iron in serum is believed to be bound entirely to transferrin (1, 2) and, to date, evidence for the existence of an ultrafiltrable fraction of iron in serum has not been published. This paper reports results of *in vitro* experiments relative to the distribution of iron in serum and presents evidence for the existence of an amino acid bound fraction of iron in human serum.

*Methods.* All solutions were prepared with ion-free water, and glassware and syringes were cleaned according to methods reported earlier (3). Pooled serum from subjects was kept frozen until used. To remove small molecular solutes, the serum was dialyzed against 4 to 6 changes of 15 to 20 times the volume of isotonic saline, adjusted to pH 7.4, in a cold room for 48 hr with constant stirring. After this treatment, the serum was designated "predialyzed serum" to distinguish it from "native serum" which was not dialyzed. Purified protein fractions such as human serum albumin[2] and transferrin[3] were also predialyzed as described above prior to their use.

Amino acids[4] were dissolved in a small volume of normal saline, usually in amounts sufficient to achieve final concentration at the upper limit of the normal physiological range in human plasma (4). The $^{59}Fe$ (ferric chloride)[5] used contained 50 mCi/ml of $^{59}Fe$ and 4 mg/ml of stable carrier iron.

The general design of the experiment was as follows: Five milliliters of native or predialyzed serum or protein solutions was placed in a test tube. After this, when applicable, appropriate volumes of amino acids were added. Labeled iron solution containing different amounts of stable iron was added in less than 0.5 ml volume. The pH was adjusted to 7.4 using an electric potentiometer with microelectrodes and either 0.1 *N* NaOH or 0.1 *N* HCl. The volume of fluid was then adjusted to 20 ml with normal saline. Samples were incubated for 30 min at room temperature. One milliliter of each sample was removed for counting the radioactivity and the remainder was subjected to ultrafiltration. The general procedure for ultrafiltration was the same as reported by Prasad and Flink (5). The cellulose casing[6] was prepared as previously described (6). Following ultrafiltration, 1 ml of the ultrafiltrate was removed for counting. The $^{59}Fe$ was counted in a well-type scintillation counter[7] and the radioactivity found in the ultrafiltrate was expressed as a percentage of the initial radioactivity in the solution prior to ultrafiltration.

All samples were analyzed in duplicate. Each group of samples included a native serum control, a predialyzed serum control, and a standard containing $^{59}Fe$ in saline only, which was subjected to ultrafiltration. Every experiment was repeated three or more times.

In some experiments, concentrated dialysate, rather than an amino acid solution, was added back to the predialyzed serum. The concentrated dialysate was prepared by saving all of the dialysate from the serum dialysis, evaporating it to dryness in a flash evaporator, and taking up the dried material in a small volume of deionized water. The resulting concentrated solution was added proportionately to the serum sample. In other experiments, some of the concentrated dialysate was dried in a platinum dish at 95°,

[1] Supported by a Detroit General Hospital Research Corporation Grant.

[2] Cutter Laboratories, Berkeley, CA.

[3] Immunology, Inc., Glen Ellyn, IL.

[4] Calbiochem, Inc., Los Angeles, CA.

[5] Mallinckrodt Nuclear, St. Louis, MO.

[6] Union Carbide, Chicago, IL. (av pore size, 24A).

[7] Nuclear Measurements Corporation, Indianapolis, IN.

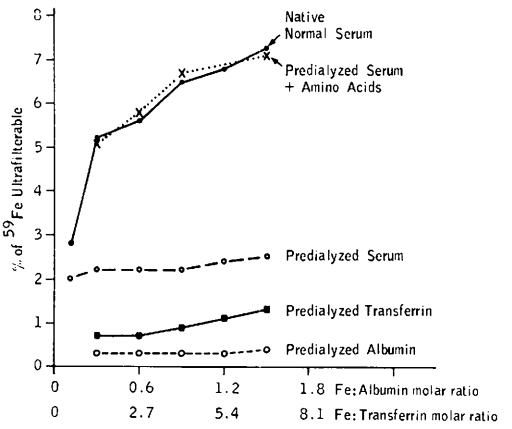


FIG. 1. Percentage of ultrafiltrable $^{59}Fe$ plotted against the different iron and protein molar ratios.

then ashed in a muffle furnace at 300°, for 40 hr. The residue was cooled and taken up in a small volume of water and added back to predialyzed serum in proper proportion.

The effect of amino acids on the ultrafiltrability of iron was studied by adding all 23 free amino acids normally present in serum in amounts necessary to achieve normal concentration (6).

*Results and Discussion.* $^{59}Fe$ in saline was 92 to 95% ultrafiltrable, indicating that there was no selective adsorption of iron on the membrane or gauze used in these experiments. The coefficient ot variation for ultrafiltrable $^{59}Fe$ throughout our studies was less than 8%. Figure 1 shows that the percentages of ultrafiltrable $^{59}Fe$ in cases of predialyzed albumin, transferrin, and serum remained low at different concentrations of iron. Considerably more $^{59}Fe$ was ultrafiltrable when native human serum was used.

The percentages of ultrafiltrable $^{59}Fe$ at different levels of added iron became similar to that of the native human serum after addition of physiological amounts of 23 amino acids normally present in serum to predialyzed serum. Figure 2 shows that dialysis of the serum before addition of $^{59}Fe$ led to a marked reduction of the percentages of ultrafiltrable $^{59}Fe$ when the iron:albumin molar ratio was 0.9. A marked increase in ultrafiltrable $^{59}Fe$ was observed when the dialysate was added back to the predialyzed serum. However, when the ashed dialysate was used to reconstitute the predialyzed serum, the iron binding property of the predialyzed serum was not altered, suggesting that the effective constituents of the dialysate of serum were organic in nature.

In other experiments, addition of histidine alone ($1.4 \times 10^{-4}$ *M*) to predialyzed serum raised the percentage of ultrafiltrable $^{59}Fe$ from 2.2 to 2.8% when iron:albumin molar ratio was 0.9; whereas addition of lysine ($2.1 \times 10^{-4}$ *M*), resulted in an increased percentage of ultrafiltrable $^{59}Fe$ from 2.2 to 3.2%. Addition of histidine, glycine, threonine, cysteine, and lysine, in combination, at physiological concentrations, to predialyzed serum (at iron:albumin molar ratio of 0.9) increased the ultrafiltrability of iron from 2.2 to 4.5%.

Table I shows a progressive increase in the ultrafiltrability of $^{59}Fe$ when increasing amounts of amino acids were added to predialyzed transferrin and albumin and compared to their initial levels. This increase was more pronounced in the case of albumin than that of transferrin. Inasmuch as counting error in our experiments was less than 5% these differences are significant. The percentage of ultrafiltrable $^{59}Fe$ did not increase proportionately to the change in amino acid concentration, most likely due to a change

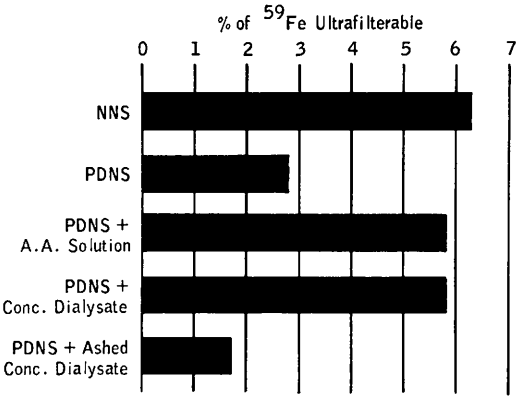


FIG. 2. Percentage of ultrafiltrable $^{59}Fe$ in native normal serum (NNS), predialyzed serum (PDNS), predialyzed serum with 23 amino acids (AA) added centrated dialysate (PDNS + conc. dialysate), tion), predialyzed serum reconstituted with concentrated dialysate (PDNS + conc. dialysate), and predialyzed serum reconstituted with ashed concentrated dialysate at FE:albumin molar ratio of 0.9.

TABLE I. Effect of Addition of Amino Acid Solutions to Predialyzed Protein on Ultrafiltrability of $^{59}Fe$.[a]

| | % $^{59}Fe$ ultrafiltrable | |
|---|---|---|
| | 0.3% Transferrin[b] | 4.0% Albumin[c] |
| Predialyzed | 1.3 | 0.3 |
| Predialyzed + 1AA | 1.9 | 1.1 |
| 2AA | 2.0 | 1.8 |
| 3AA | 2.3 | 2.0 |

[a] All 23 amino acid solutions (AA) were added to predialyzed protein fraction in increasing amounts; 1AA = amount normally present in serum, 2AA = twice the amount normally present in serum, and 3AA = three times the amount normally present in serum.

[b] Fe:Transferrin molar ratio = 4.0.

[c] Fe:Albumin molar ratio = 0.9.

in binding power of amino acids as a result of amino acid–protein interaction.

In our experiments, ultrafiltration of $^{59}Fe$ incubated sera was performed to determine the nonprotein bound fraction of iron inasmuch as the concentration of stable iron in the ultrafiltrate was considered to be too low to be measured accurately by existing methods. Our studies show that a small but significant portion of ultrafiltrable iron is bound to amino acids. Among other trace elements, copper (7) and zinc (6) have been reported to exist in serum as amino acid bound fraction in small quantitities. These findings for copper and zinc were confirmed by computation from stability constants and plasma composition data (8). The amino acid bound fraction of iron may have an important physiological role in the biologic transport of this element across cellular membranes. Although such a fraction was demonstrated in our experiments *in vitro,* its existence *in vivo* remains to be documented. Since a small but consistent fraction of iron was ultrafiltrable following incubation of $^{59}Fe$ with predialyzed serum, it appears that in addition to an amino acid bound fraction, free iron also constituted a small segment of ultrafiltrable iron.

After single amino acids and $^{59}Fe$ (ferrous sulfate) were introduced into isolated loops of small intestine, Drow *et al.* (9) found an an increased radioactivity in the serum and liver of rats. Brown and Rother (10) reported the existence of an amino acid bound mucosal iron complex in experimental animals and suggested that the absorption of iron from the bowel mucosa may be controlled to some extent by this complex. Charlton *et al.* (11), however, found no evidence for the presence of iron complex to amino acids in the bowel mucosa of rats. Although our studies do not deal with the mechanism of iron absorption from the bowel mucosa directly, one may consider that a possible effect of the presence of amino acids in the gut may have been to render iron more diffusible, thus facilitating its passage through the mucosal wall to some extent.

*Summary.* Following incubation of $^{59}Fe$ with pooled native human serum *in vitro,* ultrafiltrable iron was determined to be 5 to 7.5% of the total serum iron, at different levels of iron:albumin molar ratios. Under similar conditions approximately 2% of iron was ultrafiltrable when predialyzed serum was used. In physiological concentrations, additions of amino acids to predialyzed serum increased ultrafiltrable $^{59}Fe$ to similar levels as that observed for native human serum. Histidine and lysine showed significant effects in this regard. Amino acids competed effectively with albumin and only to a lesser degree with transferrin for binding of iron, suggesting a tighter affinity of iron for transferrin.

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1. Wintrobe, M. M., "Clinical Hematology," 6th ed., p. 133. Lea & Febinger, Philadelphia (1967).

2. Beutler, E., *in* "Iron Metabolism" (F. Gross, ed.), p. 256. Springer-Verlag, Berlin (1964).

3. Prasad, A. S., Oberleas, D., Wolf, P., and Horwitz, J. P., J. Clin. Invest. 46. 549 (1967).

4. Stein, W. H., and Moore, S., J. Biol. Chem. 211, 915 (1954).

5. Prasad, A. S., and Flink, E. B., J. Appl. Physiol. 10, 103 (1957).

6. Prasad, A. S., and Oberleas, D., J. Lab. Clin. Med. 76, 416 (1970).

7. Nuemann, P. Z., and Sass-Kortsak, A., J. Clin. Invest. 46, 646 (1967).

8. Hallman, P. S., Perrin, D. D., and Watt, A. E.,

Biochem. J. **121**, 549 (1971).

9. Drow, D., Kinney, T. D., Kaufman, N., and Klavins, J. V., Blood **21**, 546 (1963).

10. Brown, E. B., and Rother, M. L., J. Lab. Clin. Med. **62**, 357 (1963).

11. Charlton, R. W., Jacobs, P., Torrance, J. D., and Rothwell, T. H., J. Clin. Invest. **44**, 543 (1965).

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