Method for Determining Iron-Binding Capacity of Serum.* (22440)

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(Introduced by Edwin L. Smith.)

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The availability of radioactive iron has permitted laboratory evaluation of hematopoietic disorders based upon the rate and quantity of iron transport by the serum, and the subsequent distribution of this iron(1,2). To interpret the data obtained from iron turnover studies, it must be established that the capacity of the serum to bind iron is not a limiting factor by demonstrating residual iron-binding capacity. The serum iron concentration and residual binding capacity of normal persons and of patients with a variety of diseases have been determined using several published chemical and isotopic procedures(3-5). The simplicity of the method described here for analyzing for serum iron-binding capacity has permitted this laboratory to obtain reproducible values on a large number of samples. Iron labelled with a radioactive tracer is added to a serum sample in excess of the binding capacity, and that which becomes attached is separated by protein precipitation and measured isotopically. Preliminary studies indicated several difficulties. Ionic iron will not remain in solution at neutral pH, but precipitates as ferric hydroxide, which has a solubility product ($k_{s.p.}$). of 1.1×10^{-36} (18°C). Several authors have advocated addition of iron chloride in acid solution directly to serum to obtain protein-bound iron, since the buffering capacity of serum is adequate to neutralize small volumes of weakly acid solutions. If this is done, the iron is not dialysable, and it precipitates with the protein fraction. Nevertheless, much of the added iron is in the hydroxide form, and will not permit determination of iron-binding capacity, nor behave as a tracer for protein-bound iron *in vivo*. Iron complexes which are stable at neutral pH may be used, but the complex must not be so stable as to inhibit transfer of iron to the iron-binding protein fraction of serum (IV-7, Cohn). A preliminary study indicated that iron will be distributed between equal quantities, by weight, of citrate and of iron-binding protein in a ratio of one to seventy. Gluconate binds iron somewhat more firmly than does citrate, and versenate has even greater affinity for iron than does the protein. The most satisfactory agent was found to be ascorbic acid in small concentration. To separate the serum proteins, ammonium sulfate is the precipitating agent of choice(4). The colloidal suspension is easily precipitated by ethanol, facilitating separation by centrifugation. Washing the precipitate is not necessary, since isotope studies have shown that less than 0.1 ml of the supernatant containing less than 4% of the excess iron is occluded. Filtration is laborious and time consuming, and often subject to errors of adsorption. Heparinized plasma cannot be used in place of serum, as there is apparently an interference with iron transfer causing false low iron-binding capacity values to be obtained.

Method. A. *Reagents*: (1) Carrier-radioiron chloride solution: $Fe^{59}Cl_3$ in HCl is diluted to approximately 0.2 μc/ml. Reagent grade $FeCl_3 \cdot 6H_2O$ is dissolved in the solution to give a total iron concentration of about 60 μg/ml, and standardized against a solution made from iron wire dissolved in HCl. This solution may be stored in polyethylene bottles. If iron dissolved in HCl is used instead of $FeCl_3$ crystals, the total acidity of the final preparation (solution #2) must not exceed the buffering capacity of the serum. (2) Carrier-radioiron ascorbate solution: About 10 mg of pure crystalline ascorbic acid is dissolved in 1.0 ml of solution #1 just prior to use. (3) Saturated ammonium sulfate solution: 530 g of reagent grade $(NH_4)_2SO_4$ is dissolved in slightly less than

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one liter of iron-free water. If pH is less than 6 it is adjusted to 6.0 with saturated NaOH solution, and diluted to a final volume of one liter. The pH of this solution must be checked occasionally since it may drop due to loss of NH_3 on long standing. (4) 95% ethanol. B. *Procedure*: New glassware is maintained free from iron by washing with a detergent, rinsing once thoroughly in tap water and finally twice with distilled water. *Technic*. 1. Measure 0.1 ml radioiron ascorbate solution into a pyrex test tube 15 x 125 mm. 2. Add 1 ml serum and mix well. Allow to stand for 10 minutes at room temperature and determine the radioactivity. 3. Add 10 ml of $(NH_4)_2SO_4$ solution, mix by shaking, and allow to stand for one hour. 4. Add 1 ml ethanol, invert several times and centrifuge for 10 minutes at about 1400 x g. 5. With a fine glass stirring rod, gently tilt the protein plug and while holding it against the wall of the tube, carefully pour off supernatant. Push the plug to the bottom of the tube and loosen any precipitate adhering to the walls of the tube with the stirring rod, then rinse down the rod and walls of the tube with 1 ml distilled water. 6. Count the precipitate and calculate the iron-binding capacity according to the formula: $\frac{\text{Counts of precipitate}}{\text{Original counts added}} \times \mu g$ Fe added x 100 = $\mu g\%$ binding capacity.

The method was evaluated as follows: 1. Tagged iron was added to one ml samples of pooled patient sera in successive increments of 0.5 μg, allowed to incubate 10 minutes after which 6 μg of nonradioactive iron as ascorbate was added, and the radioiron in the precipitated protein of each was measured. Results of this experiment show that the radioiron added to the unsaturated protein remains attached during the test. Thus, on adding 0.50, 1.00, 1.50, 2.00, 2.50 and 3.00 μg of Fe ascorbate to the serum, 0.47, 0.93, 1.40, 2.03, 2.30 and 2.37 μg were found in the protein plug. In the last 2 experiments the serum was saturated with iron. 2. Pooled patient sera samples were saturated with nonradioactive iron ascorbate, and radioiron ascorbate added thereafter to prove that appreciable exchange does not take place between bound iron and unbound iron during the procedure. Upon adding 0.50, 2.50 and 5.00 μg of radioactive Fe ascorbate to the serum, 0.04, 0.18 and 0.26 μg of radioactive Fe were measured in the protein plug. 3. Graded increments of nonradioactive iron ascorbate were added to pooled sera samples, and residual iron-binding capacities measured. In this series, in which the original iron-binding capacity was 296 μg %, the addition of 50, 100, 150, 200, 250 and 500 μg % of cold Fe ascorbate left residual binding capacities which were less than the original value by 46, 103, 153, 206, 246 and 274 μg % respectively.

Summary. 1. A simple radioisotopic method is described for determining the iron-binding capacity of serum. 2. In principle, iron labelled with a radioactive tracer is added to a serum sample in excess of the binding capacity, and that which becomes attached is separated by protein precipitation and measured isotopically. 3. The method was evaluated by measurement of: (a) the recovery of radioiron added in increments to serum samples, (b) the amount of iron exchanged between the saturated iron proteinate and the excess iron in solution, and (c) the difference in residual binding capacities from the original value after the addition of increments of nonradioactive Fe ascorbate to serum samples.

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