A gel filtration method for studies on protein iron-binding*

This communication reports the use of a gel filtration technique and radioactive iron for rapid determination of protein-iron binding in complex mixtures. Many methods have been used to investigate the characteristics of iron binding by mammalian serum. Colorimetric techniques have been used to measure the iron-binding capacities of whole sera[1]. Electrophoretic[2,3] and precipitation[4,5] methods, using radioactive iron (^{59}Fe), have confirmed the role of a β-globulin, transferrin, as the primary iron-binding component of serum. Each of these techniques has certain limitations regarding specificity, binding interference, and rapidity of sample processing.

Filtration through cross-linked dextran gels separates components of a complex mixture on the basis of molecular dimensions[6-8]. The technique has been used for studies of the binding between proteins and small molecules[9,10].

EXPERIMENTAL

In the present system, protein-bound and non-protein bound iron were separated on 25 × 1.5 cm columns of Sephadex G-50, except where otherwise indicated. Rat (Sprague-Dawley adult males) serum samples, containing radioactive iron, were added to the column. The samples were eluted with buffer using a flow rate of 90 ml/h established by a Milton Roy Minipump. The effluent passed through P.E. 190 intramedic polyethylene tubing (Clay Adams) which was coiled around the base of a 10 × 100 mm test tube. This coil was inserted into a well-type scintillation counter. The radioactivity of the effluent was continuously monitored on a rate meter and recorded directly on a strip chart recorder. The protein content of the effluent was monitored in the initial experiments using a quartz flow cell as described by ANDERSON[11]. Protein monitoring was discontinued since equivalent protein elution occurred with uniform effluent volumes. Volume measurements were recorded in each experiment.

Except where otherwise indicated, serum samples were prepared by combining the following: 0.1 ml of a 1.0 μC/ml solution of ^{59}Fe (specific activity 42.5 mC/mg); carrier iron ($FeCl_2 \cdot 4H_2O$) in excess of the unsaturated iron-binding capacity (UIBC) of the serum; 0.5 ml of serum; and citrate to a final concentration of 0.001 *M*. The volume was brought to 1 ml with the eluent buffer and 0.50- or 0.75-ml aliquots were added to the column. Citrate prevented attachment of non-protein bound iron to the gel, but did not affect protein-iron binding as has been previously reported[4].

Better separations of the protein- and non-protein bound iron were obtained with Sephadex G-50 than with Sephadex G-25 (Fig. 1). The first peak of radioactivity eluted contained protein-bound iron, whereas non-protein bound iron was found in the second peak. Experiments at pH 5.0 and 7.0 illustrate the use of this technique for quantitatively determining iron binding by the protein (Fig. 2). Little iron was bound at pH 5.0, whereas at pH 7.0 a considerable amount was bound. UIBC values were determined from these recordings by measuring the percentage of the total area under each peak (height × width at half-height). At pH 7.0, for example, 42.2% of the 3 μg of iron in 0.5 ml serum was bound to protein. All iron binding was then calculated in terms of 100 ml of serum and expressed as "μg%". The amount of iron

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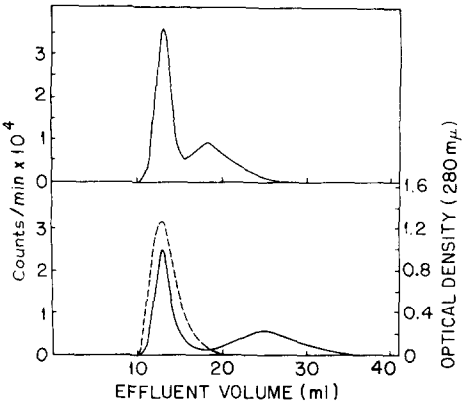

Fig. 1. Separation of protein bound and non-protein bound iron by gel filtration through Sephadex. Column dimensions, 50 × 0.9 cm; flow rate 90 ml/h; elution buffer was 0.01 *M* Tris–citrate, 0.15 *M* NaCl, pH 7.0 ——— radioactivity – – – – O.D. at 280 mμ. Different rat serum samples were used for each experiment.

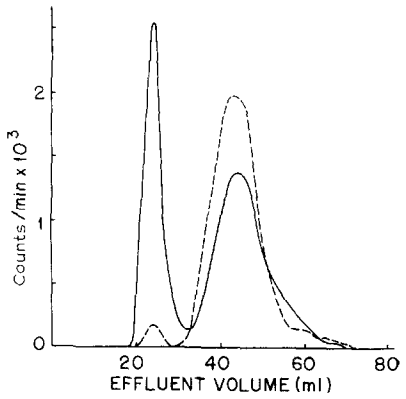

Fig. 2. Protein-iron binding at pH 7.0 and pH 5.0. Sephadex G-50; column dimensions, 25 × 1.5 cm; flow rate, 90 ml/h; elution buffer was 0.01 *M* Tris–acetate, 0.15 *M* NaCl, 0.001 *M* citrate pH 7.0 ——— and 5.0 – – – –.

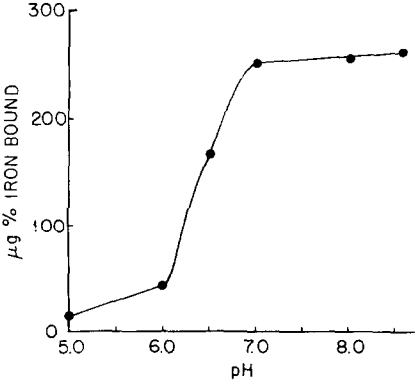

Fig. 3. Effect of pH on protein-iron binding. Sephadex G-50; column dimensions, 25 × 1.5 cm; buffer was Tris–acetate 0.01 *M*, 0.15 *M* NaCl, 0.001 *M* citrate adjusted to the pH required. For calculations of μg% of iron bound, see text. Serum was dialyzed against appropriate buffer before the samples were prepared for application to the column.

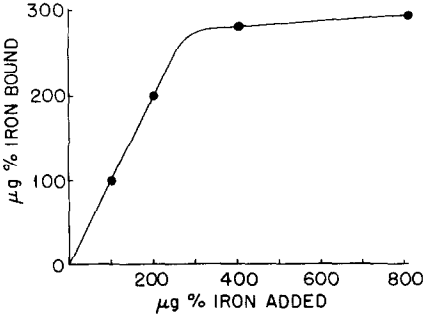

Fig. 4. Protein-iron binding as a function of the amount of iron added. Sephadex G-50; column dimensions 25 × 1.5 cm; flow rate 90 ml/h; buffer was 0.01 *M* Tris–acetate, 0.15 *M* NaCl, 0.001 *M* citrate, pH 8.5.

bound at pH 7.0 was 253 μg%, which corresponds to colorimetrically determined values.

The UIBC of serum proteins as a function of pH is illustrated in Fig. 3. Iron binding was maximal above pH 7.0 and decreased greatly below this value. This curve is shifted approximately 0.5 pH units to the right of other published curves[4]. This shift was noted only in predialyzed serum samples. This effect of dialysis on serum protein-iron binding is currently being investigated.

At pH 8.5, serum proteins bound 100% of the added iron until saturation of the protein occurred (Fig. 4). A slightly rising plateau was noted when iron was added in

excess of the UIBC. These results agree with colorimetric determinations[5] but differ from precipitation studies on iron binding as a function of iron added in that 100% of the iron was bound when iron was added in amounts less than the UIBC of the protein[5]. Also, no plateau was attained when the amount of added iron exceeded the known saturation values of the serum proteins. The lower iron-binding values obtained by protein precipitation methods could have resulted from a slight dissociation of protein-bound iron. The lack of a plateau was apparently due to the precipitation of non-protein bound iron, both in the presence and absence of citrate[5]. No evidence for precipitation of colloidal iron on the gel was observed with the concentrations of citrate used here.

It may be concluded that the gel filtration technique, combined with procedures for continuous monitoring of radioactivity, provides a rapid and quantitative method for the direct analysis of protein-iron binding in complex mixtures. The method has been tested using serum protein-iron binding as a model system and is now being used routinely in our laboratory for analyzing iron binding by soluble proteins of tissues.

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Haptoglobins in pleural and ascitic fluids

As part of a study of the biological and clinical significance of haptoglobins, we have measured haptoglobin concentrations in pleural and ascitic fluids in various pathological conditions and have compared the haptoglobin concentrations in such fluids with those in plasma.

Specimens of fluid (35 pleural and 15 ascitic) from 50 Caucasian patients were