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A Radioimmunoassay for Duck Serum Ferritin

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Development and optimization of a radioimmunoassay for duck serum ferritin are described. ^{125}I-labelled ferritin and rabbit anti-ferritin antibody are used together with goat anti-rabbit gamma globulin as separating agent for the bound and free fractions. The assay has a working range of up to 500 μg of ferritin per litre and a sample requirement of 50 μl of serum. The assay requires a 24 h period and has a sensitivity of 10 μg of ferritin per litre.

Key words: Duck, Radioimmunoassay, Serum ferritin.

The finding of that ferritin was not exclusively intracellular, but it could be found in serum, reported by MAZUR and SHORR (13), was unimportant until ADDISON *et al.* (1) showed that in human serum ferritin concentration correlates iron stores status, in iron deficiency and iron overload.

After the publication of the IRMA procedure for serum ferritin, many workers have carried on developing and performing new RIA and IRMA procedures to measure more accurately serum ferritin levels and their variations with some diseases affecting the iron metabolism, in order to extend this correlation (6, 11, 14, 15). It has been made in other animal species too, as rat and chicken, by feeding them iron-loaded and iron-deficient diets (2, 8, 17).

The study of this correlation in physiological conditions becomes easier in birds, where a change of about five times in serum iron concentration occurs during laying period (16), as a physiological response to the egg production needs. Treatment with estrogens mimetizes the laying period and depletes iron stores to account for serum iron increase, recovering their normal values after the estrogen effect has passed (12).

Serum ferritin in birds has only been measured in hen (2); for this reason the purpose of the present work is to develop a RIA procedure for duck serum ferritin to make possible the measurement of serum ferritin concentration in relation to iron stores status.

Materials and Methods

Duck liver ferritin and rabbit antibodies against it were prepared as described previously (4).

Radioiodination of duck liver ferritin. Ferritin was labelled with I-125 by the chloramine T procedure of HUNTER and GREENWOOD (7). Protein, 10 μg in 10 μl of phosphate buffer (0.1 mol/l, pH 7.4) was added to 0.5 mCi of I-125 in 10 μl (200 Ci/l, Amesrshan) diluted with an equal volume of phosphate buffer; 10 μl of chloramine T (4 g/l) was added and after 30 s at room temperature, 20 μl of sodium metabisulfite (6 g/l) was added. After addition of 500 μl of KI (2 g/l), the labelled ferritin was separated from iodide free by use of a column containing Sephadex G-10 (1 × 20 cm), preequilibrated with phosphate buffer (0.1 mol/l, pH 7.4, with added bovine albumin, 2 g/l). Elution was monitored with a gamma-counter Packard 500 and the iodinated ferritin peak was checked as is described in *Procedure.*

The iodinated ferritin was diluted in phosphate buffer with added bovine albumin so that 100 μl yielded about 50,000 cpm. The labelled material undergoes a slow loss of immunoreactivity, with a half life of about three weeks.

Goat anti-rabbit gamma-globulin. Anti-rabbit gamma-globulin antibodies were induced by injecting a male goat subcutaneously with rabbit gamma globulin (Miles Martin), 5 mg in 5 ml saline and 5 ml Freund's adjuvant were given six times at weekly intervals. A week after the second injection the appearance of antibodies was checked by Ouchterlony-type immunodiffusion.

Procedure. The procedure described here is based on that proposed by LUXTON *et al.* (11): 100 μl of anti-ferritin antibody diluted in phosphate buffer (0.1 mol/l, pH 7.4, with normal rabbit serum 1 ml/l, disodium ethylenediamine tetraacetate, EDTA, 20 g/l, and NaCl 9 g/l) were incubated with 100 μl of labelled ferritin (about 2 ng) and 50 μl of buffer, standard or serum in 12×75 mm tubes overnight at room temperature. Bound and free fractions were separated by addition of 100 μl of goat anti-rabbit gamma-globulin (10-fold dilution). After mixing the contents and allowing the tubes to stand at room temperature for 5 h, the tubes were centrifuged at 1,500 × *g* for 30 min. The supernatant (free) fraction was aspirated and the precipitated (bound) fraction counted.

Antibody dilution. A series of tubes was prepared containing labelled ferritin diluted to give 55,000 cpm and with dilutions of first antibody from 1 to 10^4 to 1 to 1.28×10^6. After incubation overnight at room temperature, second antibody, diluted 10-fold in phosphate buffer (0.1 mol/l, pH 7.4), was added and after subsequent incubation at room temperature for 5 h and centrifugation, the centrifuged was counted.

Standard curve. Using first antibody at a dilution of 1.6×10^5, labelled ferritin 55,000 cpm, and a range of standards from 1 to 500 μg/l; a standard curve was set up with first incubation overnight at room temperature, followed by incubation with second antibody 10-fold diluted, for 5 h at room temperature.

Duck liver ferritin concentration was measured by the method of LOWRY *et al.* (10), using a standard solution prepared from bovine albumin (Sigma). Ferritin concentration was 1,292 ± 26 mg/l (n = 9). Standards were prepared by diluting this solution in phosphate buffer (0.1 mol/l, pH 7.4, with added bovine albumin 2 g/l) to give concentrations of 1, 2.5, 5, 10, 20, 25, 50, 100, 150, 250 and 500 μg/l.

Scatchard's plot. A standard curve was set up using first antibody at a dilu-

tion of 1 to 150,000, labelled ferritin 25,000 cpm, equivalent to 3.3 ng, and a range of standards from zero to 500 μg/l. In addition, three zero standard tubes were set up with incremental increases of labelled ferritin: 42,000, 87,000 and 119,000 cpm. These three tubes must contain the regular mass of labelled ferritin present in every standard tube with additional mass increments of one, three and five times the mass present in 25,000 cpm. The corresponding values (y_1, y_3, y_5) read off the standard curve gave results (Ag_1, Ag_3, Ag_5) in terms of unlabelled ferritin concentration. When divided by 1, 3 and 5, respectively, these values yielded 31, 30 and 26 μg/l, as estimates of labelled ferritin concentration, Ag*. The coincidence of these values indicated that antibody was not discriminating between labelled and unlabelled ferritin. With Ag* defined and y values for each added standard Ag_1, the binding capacity, Ab, and equilibrium constant, K can be evaluated by using a Scatchard's plot with Bound/Free = y/(1 — y) as ordinate and Bound = y(Ag* + Ag_1) as abcissa.

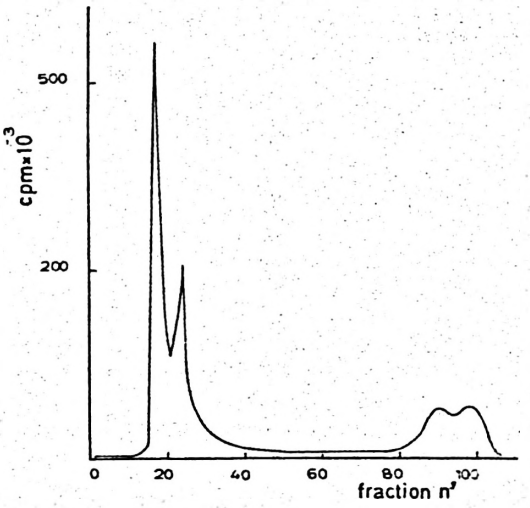

Fig. 1. *Elution profile from Sephadex G-10 after radioiodination of ferritin by the chloramine T method.*
It has been set up with 10 μl aliquots from collected fractions. First peak corresponds to labelled ferritin.

Results

Iodination of ferritin. Iodinated ferritin was separated from iodide free by gel filtration on a Sephadex G-10 column (fig. 1). The first peak (fractions 15 to 22) corresponds to labelled ferritin, 90 % of its radioactivity was precipitable by 10 % trichloroacetic acid, and 85 % of the iodinated ferritin could be recognized by an excess of the proper antibody. Efficiency of iodination was usually about 10 %, as calculated from the radioactivity recovered from the first peak and total radioactivity used for labelling, yielding labelled ferritin with a specific activity ranging between 4.0 and 7.0 μCi/μg of ferritin. Iodinated ferritin, diluted in phosphate buffer with added bovine albumin, becomes stabilized and remains

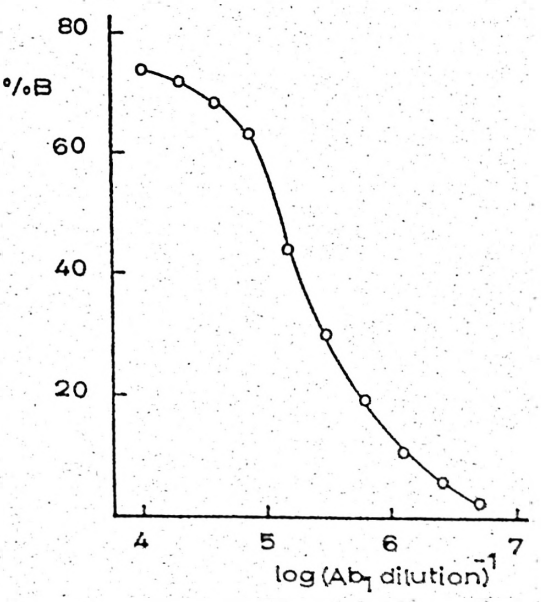

Fig. 2. *An antibody dilution of 1 to 160,000 binds 50 % of labelled ferritin.*

usable for as long as three weeks in the RIA for ferritin.

Antibody dilution. Plotting y = true bound counts/total counts available for binding against log (Ab dilution)$^{-1}$, figure 2, an antibody dilution of 1 to 160,000 had a y value closest to 0.5 and this dilution was selected for posterior studies.

Standard curve. Fig. 3 was obtained plotting % B against ln(unlabelled ferritin concentration). The sensitivity of the assay was 10 μg/l, defined as the minimum amount of unlabelled ferritin statistically different from zero. The standard curve was linear in the range of 10 to 500 μg/l, and typical coefficient of variation obtained from four determinations was less than 5 %.

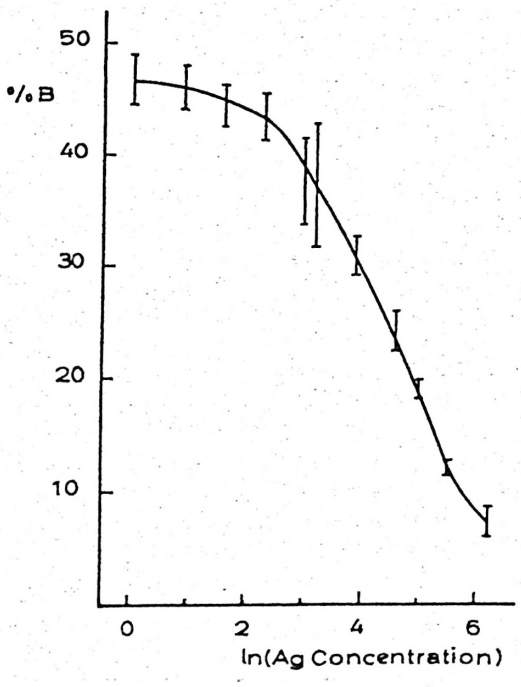

Fig. 3. *Ferritin radioimmunoassay standard curve.*
Error bars indicate standard deviation of n=9 (from 1 to 10 μg/l) and n = 4 (from 10 to 500 μg/l) determinations.

Non-specific binding optimization. The effect of normal rabbit serum and EDTA concentration in the antibody dilution buffer and second antibody amount on separation of free from bound ferritin was measured using variable relative concentrations of these compounds. These concentrations were tested in two extreme situations: non-specific binding and complete binding of labelled ligand. A second antibody dilution of 10-fold, EDTA concentration of 20 g/l and an amount of normal rabbit serum 0.1 % were selected as providing maximal discrimination between bound and free.

Scatchard's plot. Labelled ferritin concentration of 30 μg/l is nearly coincident with that which could be calculated from labelling values after gel filtration (33 μg/l). From these values it can be deduced that any important experimental mistake has not been committed; that all the radioactivity recovered from the first peak of Sephadex G-10 column was really highly purified labelled ferritin, and that the antibody was not discriminating between labelled and unlabelled ferritin. Therefore, the immunological properties of ferritin have not been disturbed by the labelling process.

From figure 4 it can be deduced that the present antibody at 1 to 1.6×10^5 dilution had a binding capacity Ab = = 12 ng/ml, and $K^{-1} = 0.17$ ng/ml, or $K = 2.7 \times 10^{12}$ 1/mol. The linear relationship indicates a homogeneous species of binding sites over the concentration range used.

It has been shown by EKINS *et al.* (5) that the theoretical optimum sensitivity occurs when $Ab = 3K^{-1} = 18$ ng/ml and $Ag^* = 4K^{-1} = 24$ ng/ml. As the results obtained were Ab = 12 ng/ml and $Ag^* = 30$ ng/ml, and the estimate of K^{-1} is always subject to considerable imprecision, it can be concluded that if the

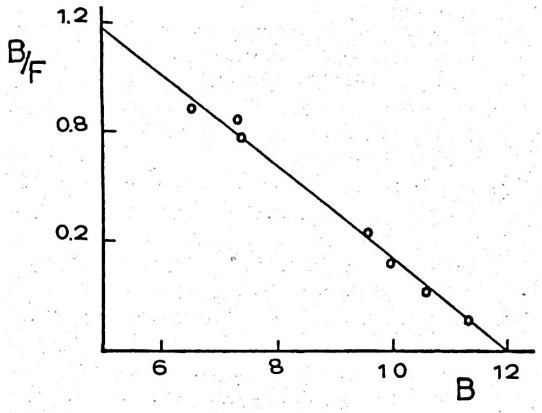

Fig. 4. *Scatchard's plot of ferritin standard curve.*
Ordinate: $B/F = y/(1-y)$; abcissa: $B = y(Ag^* + Ag_1)$.

measuring conditions are not optimum they are very close to it.

Serum dilution. 1/50 dilution was selected for duck serum ferritin measures as providing concentrations within the range of the standard curve. In order to test matrix effects of serum dilutions, three serum samples were assayed with the use of four different aliquots of each sample (20, 40, 60 and 80 µl). The results (not shown), when corrected for dilution, were identical within the confidence limits of the assay.

Discussion

Sensitivity of an assay is closely related to the affinity, or K value, of the antibody and, in general, the antiserum of higher K value, as indicated by a Scatchard plot, must be selected. This applies even with those assays where maximal sensitivity is not a requirement, since a high K value implies, although does not guarantee, a fast association constant and thus rapid attainment of equilibrium (3). From the Scatchard plot (figure 4), affinity constant K was estimated on about 2.7×10^{12} l/mol. LUXTON *et al.* (11) obtained antibodies with K value of 3.7×10^{11} l/mol and, in practice, the K values of antibodies in commonly used RIA ranges from 10^9 to 10^{12} l/mol (3). Taking as a very approximate rule of thumb the sensitivity equivalent to K^{-1}, the detection range for the RIA presented here could be extended to 0.17 ng/ml. Although detection limit really measured was 1 ng/ml, it was necessary to desensitize the assay because of high duck serum ferritin concentration.

Although antisera titre does not directly reflect affinity, antisera with higher titres would be preferred because they would yield more assay tubes/ml of the raw serum. The titre of the antiserum used in the present work was about 160,000, similar that achieved by LUXTON *et al.* (11) and clearly higher than that of CALVO *et al.* (2).

Sensitivity of an assay is also related to the specific radioactivity of labelled antigen. Substitution of one atom of I-125 per molecule of antigen can be regarded as the optimum (3) and this rule has been followed in all labelled ferritins used as antigen in the RIA exposed here; higher levels may both alter the immunoreactivity of the antigen and also increase the likelihood of decay catastrophe, causing a life span shortened.

Sample serum dilution, although increases sample manipulation and is timecomsuming, avoids measuring imprecision of the small volume of serum that it would be necessary without dilution.

Above mentioned features allow the RIA method explained to evaluate duck serum ferritin concentration with a high degree of accuracy and precision that turn it into a valuable method for the measuring of duck serum ferritin levels.

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Resumen

Se describe el desarrollo y la puesta a punto de un método de radioinmunoanálisis para ferritina sérica de pato, usando ferritina marcada con I-125 y anticuerpos de conejo antiferritina de hígado de pato, junto con anticuerpos de cabra antigammaglobulinas de conejo como agente separador de las fracciones ligada y libre. El análisis permite trabajar con concentraciones de hasta 500 μg/l y necesita 50 μl de suero para su realización, tiene una sensibilidad de 10 μg/l de ferritina y requiere 24 h para la obtención de los resultados.

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