

# An immunoradiometric assay for ferritin in the serum of normal subjects and patients with iron deficiency and iron overload

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**SYNOPSIS** An immunoradiometric assay for human ferritin has been developed. Concentrations of ferritin in the serum of male and female controls and patients with iron deficiency and iron overload were measured.

Male controls were found to have a significantly higher mean concentration of serum ferritin than females. Patients with iron deficiency had significantly lower levels than normals of either sex and patients with iron overload had greatly elevated serum ferritin concentrations. It is thought that the serum ferritin concentration may reflect the iron stores of the body.

Ferritin is a high-molecular weight iron compound consisting of a protein shell with a molecular weight of 450 000 and a variable amount of hydrated ferric phosphate which forms a central core within the protein shell. Iron may comprise up to 20% of the molecule which may have a molecular weight as high as 900 000. Ferritin is found mainly in the cytoplasm of reticuloendothelial cells, liver cells, and to a lesser extent in the developing red cell precursors in the bone marrow. It has normally been considered as a storage compound from which iron is readily mobilized either into the transferrin-bound plasma pool or for intracellular haem synthesis. It has not been thought to appear in the plasma or extracellular fluid under normal conditions (Reissmann and Dietrich, 1956; Aungst, 1966).

Previous methods for the estimation of ferritin have been relatively insensitive (Reissmann and Dietrich, 1956; Beamish, Llewellyn, and Jacobs, 1971) and have been able to detect ferritin concentrations in the serum corresponding to about 2.5 µg of ferritin iron per 100 ml. Using these techniques it has not been possible to detect ferritin in normal or iron-deficient sera. The present paper describes a sensitive method for measuring ferritin protein and reports the concentration in the sera of normal subjects and those with iron deficiency and iron overload.

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## Materials and Methods

A horse ferritin immunoabsorbent was prepared as previously described (Miles and Hales, 1968). One hundred mg of diazocellulose and 200 mg of horse ferritin (Koch Light Limited) were reacted for 48 hr at 4°C in the dark. The immunoabsorbent was washed 10 times in 0.15 M phosphate buffer pH 7.4 containing 9 g per litre sodium chloride. After this washing the free ferritin, as measured by OD at 430 nm of the supernatant, fell to zero. However, on storing the immunoabsorbent for several days the OD at 430 nm of the supernatant increased and the horse ferritin immunoabsorbent was always re-washed three times before use: 22.5 mg (11.25% of the horse ferritin was coupled giving an immunoabsorbent with 225 mg antigen per g cellulose.

The antibodies from 1 ml of both rabbit antihorse ferritin serum and rabbit antihuman ferritin serum were extracted separately with the horse ferritin immunoabsorbent by incubation at 4°C for three to four days. After washing six times in the phosphate-saline buffer the protein uptakes were measured by the method of Lowry, Rosebrough, Farr, and Randall (1951) using a horse ferritin standard. A control non-immune serum was also incubated with the immunoabsorbent to determine the level of non-specific uptake of protein. The specific uptake of antibody was 690 µg antihorse ferritin antibody and

270  $\mu\text{g}$  antihuman ferritin antibody per mg cellulose base: 14.4 molecules antihorse ferritin antibody and 5.6 molecules of antihuman ferritin antibody were bound per molecule antigen, assuming that all the antibody was IgG of molecular weight 160 000. The results for the horse ferritin antibody uptake by the immunoabsorbent compare favourably with those reported by Kabat (1961) using free ferritin.

Iodination of the antibody was carried out while the antibody was coupled to the immunoabsorbent. Between 50 and 100  $\mu\text{g}$  protein was iodinated using 25  $\mu\text{g}$  Choramine T (Addison and Hales, 1971), and 1.2 mc  $^{125}\text{I}$  (IMS30 Radiochemical Centre, Amersham). The labelled antibody-immunoabsorbent was washed in a filter funnel, lined with a double thickness of filter paper, with 200 ml of 0.05 M veronal buffer pH 8.0 containing 5 g per litre bovine serum albumin, 12 g per litre sodium chloride, 200 mg per litre sodium azide, and 20 mg per litre non-immune rabbit IgG. This buffer is referred to as NIRG buffer. A similar buffer with the constituents at twice the concentration of NIRG buffer was also used at times and is referred to as double-strength NIRG buffer. The low affinity antibodies were eluted with 200 ml hydrochloric acid pH 3.0. The high-affinity antibodies were then eluted with pH 2.0 hydrochloric acid, which was added in two aliquots of 4 ml and one aliquot of 100 ml. The first two aliquots were separately collected in polythene pots 75  $\times$  24 mm (Gallenkamp Limited) containing 4 ml of double-strength NIRG buffer to buffer the hydrochloric acid to pH 8.0. Two mg of freshly washed horse ferritin immunoabsorbent was added to the first two aliquots and incubated at 4°C for 24 hours. The  $^{125}\text{I}$ -antibody immunoabsorbent complex was washed three times in NIRG buffer and the uptake of labelled antibody measured. Aliquots, each of 0.5-1  $\mu\text{C}$  of  $^{125}\text{I}$ -antibody immunoabsorbent complex, were snap frozen on dry ice and stored at -20°C until required.

Human ferritin was prepared from spleens removed at operation (Beamish *et al*, 1971) and its purity was confirmed by polyacrylamide gel electrophoresis.

### Immunoradiometric Assay

$^{125}\text{I}$ -antibody was eluted from the stored aliquots after unfreezing. The  $^{125}\text{I}$ -antibody immunoabsorbent complex was washed in a 4 cm filter funnel in sequence with 30 ml NIRG buffer and 15 ml hydrochloric acid pH 3.0.  $^{125}\text{I}$ -antihuman ferritin antibody was then eluted with 2 ml pH 2.0 hydrochloric acid into 2 ml double-strength NIRG buffer.  $^{125}\text{I}$ -antihorse ferritin antibody was eluted with pH 2.5 hydrochloric acid, this fraction having been found to

give a better assay standard curve than the pH 2.0 fraction for this particular antiserum. Twenty  $\mu\text{l}$  (0.5 nc) of labelled antibody was incubated in plastic microfuge tubes (EET 23 or PRO 23, Beckman-Spinco) for 24 hours at 4°C with 50  $\mu\text{l}$  of standard or unknown solution of ferritin. Binding  $^{125}\text{I}$ -antibody to immunoabsorbent in the absence of ferritin is reduced if NIRG buffer is replaced by serum from an animal, eg, rabbit, whose ferritin does not cross react with the labelled antibody. This effect has been shown to be due to the high protein concentration, an identical effect being produced by 40 g per litre bovine serum albumin. For this reason the standard ferritin solutions were made up in 0.05 M veronal buffer pH 8.0 containing 12 g per litre sodium chloride, 200 mg per litre sodium azide, and 40 g per litre bovine serum albumin. Serum samples which required diluting were also diluted with this buffer.

Fifty  $\mu\text{l}$  of a suspension of 0.5 mg per ml horse ferritin immunoabsorbent in NIRG buffer was added to the tubes. All tubes were kept at 4°C until the immunoabsorbent was added and were then mixed for 30 min at room temperature. After spinning for 3 min in a Beckman microfuge, 90  $\mu\text{l}$  of the supernatant was removed with a 100  $\mu\text{l}$  Hamilton microsyringe (710 NCH) and counted in a Wallac Decem gamma counter.

### ANTIHORSE FERRITIN SERUM

The antihorse ferritin serum used in these experiments precipitated human ferritin and an attempt was therefore made to use this cross reaction for the assay of human ferritin. Using  $^{125}\text{I}$ -antihorse ferritin antibodies the assay was capable of detecting a concentration of 3.9 ng per ml horse ferritin ( $P < 0.001$ ). However, in the immunoradiometric assay the cross reaction observed with human ferritin was too poor to be of use in the measurement of normal or low plasma levels of human ferritin. The cross reaction could still be used in the isolation of antihuman ferritin antibodies using the horse ferritin immunoabsorbent.

### ANTIHUMAN FERRITIN SERUM

A standard curve for human ferritin is shown in Figure 1. The lowest dilution determined in this assay, 0.22 ng per ml ferritin protein, was highly significant ( $P < 0.001$ ). The actual amount of ferritin measured in the assay is equivalent to  $2.4 \times 10^{-17}$  moles human ferritin. The assay showed complete cross reaction between human liver and spleen ferritin and the effect of a reticulocyte homogenate was shown to dilute out parallel to the standard curve. Serial doubling dilutions of human serum were found to give parallel reductions in ferritin content (Fig. 2).

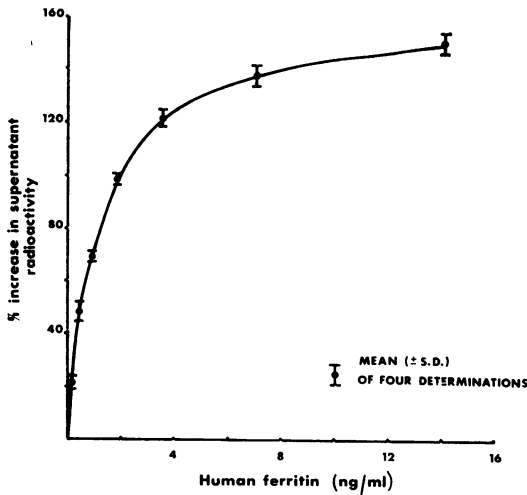


Fig. 1 Standard curve of assay of human ferritin. Total radioactivity 379 cpm: 67% of counts bound in the absence of ferritin.

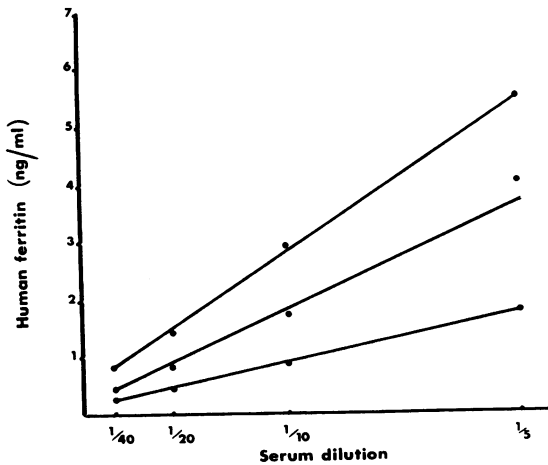


Fig. 2 Assay of ferritin concentration in successive dilutions of three serum samples.

Serum was obtained from 33 healthy male and 18 healthy female controls. Samples were also collected from 15 patients with iron-deficiency anaemia. Haematological data for these groups are shown in the Table and individual ferritin concentrations in Figure 3. The mean serum concentration of ferritin in males,  $52 \pm 5.1$  ng per ml, was significantly

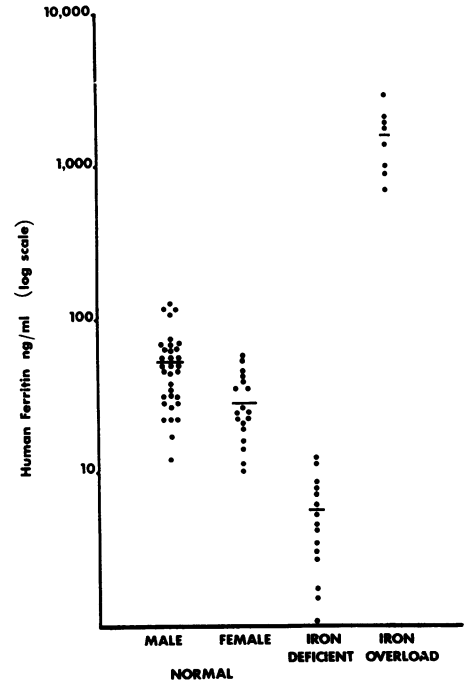


Fig. 3 Serum ferritin concentrations in male and female controls and patients with iron deficiency and iron overload.

higher than the mean concentration in females,  $28.8 \pm 3.3$  ng per ml ( $P < 0.001$ ). The sex difference in serum ferritin concentration is greater than the difference in iron status reflected by the transferrin saturation in the two sexes and is still maintained if the controls are matched for transferrin saturation

	Ferritin (ng/ml)	Haemoglobin (g/100 ml)	Serum Iron ( $\mu$ g/100 ml)	Transferrin Saturation (%)
Male n = 33	$52 \pm 5.1$ (12-128)	$15.7 \pm 0.15$ (13.4-17.9)	$120.7 \pm 5.5$ (76-206)	$30.8 \pm 1.6$ (17-54)
Female n = 18	$28.8 \pm 3.3$ (10-56)	$14.5 \pm 0.2$ (12-16)	$119 \pm 7.0$ (78-196)	$25.0 \pm 1.8$ (15-43)
Iron-deficient n = 15	$5.3 \pm 0.9$ (0.6-12)	$9.1 \pm 0.44$ (5.6-12.6)	$22.0 \pm 2.1$ (14-36)	$4.6 \pm 2.0$ (2-11)

Table Haematological profiles of male and female controls and iron-deficient patients

( $P < 0.05$ ). No correlation was observed between serum ferritin levels and any of the other three parameters.

The mean serum ferritin concentrations in 15 patients with iron deficiency,  $5.3 \pm 0.9$  ng per ml, was significantly below the levels for normal males and females ( $P < 0.001$ ). There was only a small overlap with the normal range (Fig. 3). Serum ferritin concentration was measured in one patient with haemochromatosis and eight patients with aplastic anaemia and haemosiderosis. The mean concentration was 1528 ng per ml with a range of 680 to 2800 ng per ml.

### Discussion

Previous studies have been unable to detect ferritin in serum from normal subjects (Reissmann and Dietrich, 1956; Aungst, 1966) and it has only been detectable in pathological states at concentrations in excess of  $0.04 \mu\text{g}$  ferritin iron per ml (Beamish *et al.*, 1971). It has been assumed that if ferritin is present in normal serum it is below this concentration. The sensitive assay used in the present study shows that ferritin is present in normal serum in concentrations between 10 and 100 ng per ml. The source of this ferritin in the peripheral blood can only be a matter of speculation at the present time but as the largest concentration in the body is present in reticuloendothelial cells it seems likely that this is the source. The well defined range of serum concentrations implies a fairly steady influx and this might result from either an active process of excretion by reticuloendothelial cells or from release as moribund cells reach the end of their normal life span. The difference in serum ferritin concentration in males and females is remarkable. While the delivery of iron to the bone marrow for erythropoiesis as indicated by transferrin saturations is not greatly different in the two sexes, it is known that there are marked differences in storage iron levels. Pritchard and Mason (1964), using the technique of repeated phlebotomy, found the average stores in males to be 819 mg while those of nulliparous females averaged only 254 mg. Weinfeld (1971) has calculated

the average non-haem iron content of liver to be 400 mg for men and 130 mg for menstruating women. The differences in the serum ferritin concentrations are in keeping with these known differences in storage iron level. The results of ferritin estimation in patients with iron deficiency and iron overload reinforce the suggestion that serum levels largely reflect iron stores. Patients with iron-deficiency anaemia have levels approximately one tenth those of normal subjects and this may be related to the small amounts of non-haem iron inevitably associated with the process of erythropoiesis and haemoglobin breakdown. There is a very wide separation between normal concentrations and those found in patients with iron overload. If the relationship of serum ferritin with body stores of iron is substantiated then the assay will provide a valuable method of screening subjects for subnormal or excessive storage levels.

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