Measurement of Ferritin in Serum: Application in Diagnostic Use

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A sandwich-type radioimmunoassay for serum ferritin was developed using iron-rich human liver ferritin and evaluated for its clinical usefulness. In young healthy males and females, the mean serum ferritin concentrations were 44 μg/L (range 7–158) and 16 μg/L (range 4–56), respectively. In anemic patients lower serum ferritin concentrations were found, while in most patients with iron overload serum ferritin concentrations well above 1000 μg/L were measured. Comparison of our method with a commercially available radioimmunoassay kit revealed a good correlation, except for sera with very low ferritin concentrations. Comparison with serum iron and transferrin parameters in patients with iron deficiency demonstrated that serum ferritin concentrations might be subnormal in a majority of patients with otherwise normal iron indices. Up to 70% of the ferritin in serum of normal subjects could bind to concanavalin A-Sepharose, indicating its glycoprotein nature. It is concluded that our serum ferritin radioimmunoassay gave reliable results and was useful in the laboratory diagnosis of latent iron-deficiency and in the analysis of the heterogeneity of serum ferritin.

KEY WORDS: ferritin, sandwhich-type radioimmunoassay

Tissue iron is stored inside a porous protein capsule called ferritin (1). A small amount of this ferritin can be detected in serum by sensitive immunoassay techniques. The amount of circulating ferritin has been found to correlate well with the amount of tissue iron (for reviews, see ref. 2 and 3). The precise molecular relationship between ferritin in serum and in tissues is still not resolved, however. As opposed to tissue ferritin, a major portion of serum ferritin is glycosylated (4, 5). It appears that the glycosylation of serum ferritin is a major determinant of its survival and hence its level in the circulation (6). Where this glycosylation occurs is at present unknown.

Recently, we developed a sandwich radioimmunoassay for serum ferritin based on iron-rich ferritin, isolated from human liver. This procedure has been used successfully in the study of patients with iron storage disorders (7, 8) and to monitor the venesection therapy in patients with iron overload (van Oost *et al.*, manuscript in preparation). Our assay method has been compared to three other, commercial, radioimmunoassays. All these assays were found to be different with respect to tissue ferritin specificity (9).

In this report we wish to document our serum ferritin assay in more detail, in particular to indicate how the serum ferritin results relate to the other iron indices in patients with iron deficiency. The assay was also used in the analysis of the glycoprotein nature of serum ferritin. Some of the results have been briefly reported previously (10).

Materials and methods

CNBr-activated Sepharose 4B, concanavalin A, concanavalin A-Sepharose, Sephadex G-25, and Sephadex G-75 were obtained from Pharmacia Fine Chemicals, Uppsala (Sweden). *N*-succinimidyl 3-(4-hydroxy, 5-(^{125}I)iodophenyl) propionate (Bolton-Hunter reagent) >1375 Ci/mmol, and the Ferritin Radioimmunoassay Kit were obtained from The Radiochemical Centre, Amersham (U.K.). Alpha-D-methylglucoside and bovine serum albumin were obtained from Sigma Chemical Co., St. Louis, MO (USA). Freunds Adjuvant was from Difco Labs, Detroit, MI (USA). All other chemicals were of the highest quality available.

Ferritin purification

Ferritin was isolated from human liver following a modification of the method of Penders (11). The purification procedure consisted of heat treatment of the tissue homogenate for 10 min at 80°, removal of the insoluble residue by low speed centrifugation, followed by repeated (ten times) ultracentrifugation for 180 min at 100,000 × *g*. The purity of the product was checked by polyacrylamide electrophoresis (9). The protein-containing bands stained also for iron, indicating the presence of monomeric and oligomeric ferritin. From 960 g liver tissue, 200 mg pure ferritin was isolated. The preparation contained 256 μg iron per mg protein.

Antibody production and purification

A rabbit was immunized by subcutaneous injection of 1 mg ferritin suspended in complete Freunds adjuvant.

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After 2 weeks, the animal was given a booster injection of 1 mg ferritin in 0.154 mol/L sodium chloride. The titre of the antiserum was checked by the Ouchterlony technique (12). Blood (40 mL) was collected on two occasions. The antisera were absorbed with normal human plasma and the immunoglobulin (IgG) fraction was isolated by precipitation in half-saturated ammonium sulfate. The precipitate was dissolved in saline and dialysed against 0.1 mol/L sodium borate buffer, pH 8.5. Specific antibodies were isolated by immunoabsorption using the following procedure: Purified ferritin (25 mg) was coupled to 1 g CNBr-activated Sepharose 4B according to the instructions of the manufacturer. The immunosorbent was incubated with the IgG fraction (280 mg) of the antiserum for 1 h and subsequently washed with 0.1 mol/L sodium borate buffer, pH 8.0. The specific antibodies were eluted with 3 mol/L sodium thiocyanate, pH 5.5. The protein-containing fraction (22 mg) was immediately desalted on a Sephadex G-25 column.

Antibody labelling (13, 14)

Bolton-Hunter reagent (0.5–1 mCi, 1700 Ci/mmol) supplied in benzene was dried by a gentle stream of nitrogen gas. Purified antiferritin IgG (45 μg) was added in 50 μL 0.1 mol/L sodium borate buffer, pH 8.5. The mixture was incubated at 4°C under continuous agitation for 30 min. The conjugation reaction was terminated by addition of 50 μL 0.2 mol/L glycine in 0.1 mol/L sodium borate buffer, pH 8.5. After 5 minutes, the mixture was applied onto a minicolumn of Sephadex G-25 prepared in 0.015 mol/L sodium phosphate, pH 7.4, 0.154 mol/L sodium chloride containing 2.5 g/L gelatin, and the column was eluted with this buffer. Fractions of 1 mL were collected and monitored for radioactivity. In 12 consecutive labelling experiments, the percent of incorporated radioactivity was 13 ± 8% (mean ± S.D.), which resulted in a specific radioactivity of 2.3 ± 1.6 μCi/μg (mean ± S.D.).

Radioimmunoassay procedure

This procedure is a modification of the sandwich method for serum ferritin originally described by Halliday *et al.* (15). The 10,000-fold diluted IgG fraction of the antiferritin serum (1 mL, final protein concentration 5.6 μg/mL) in 0.015 mol/L sodium phosphate, pH 7.4, 0.154 mol/L sodium chloride (phosphate-buffered saline) was incubated for 18 h at 4°C in polystyrene tubes of 1 × 7 cm. The tubes were washed four times with phosphate-buffered saline, stored at 4°C, and used within 8 weeks. Ferritin standards and sera were diluted in 0.05 mol/L Veronal, pH 8.5, 0.1 mol/L sodium chloride, 40 g/L bovine serum albumin. All sera were tested in at least two dilutions ranging from 1/2 to 1/2000, depending on the ferritin concentration. Standard and serum dilutions (100 μL) were incubated for 18 h at 4°C in the antiserum-coated tubes. The tubes were washed four times with saline. Radiolabelled antiferritin (100 μL, diluted in phosphate-buffered saline, containing 60,000 cpm) was added to the tubes and incubated for 4 h at room temperature. The tubes were washed three times in 0.154 mol/L sodium chloride and the tube-associated radioactivity was counted. The assay response was linear up to 20 μg/L ferritin. The regression equation of a typical standard curve was y(cpm) = $428x$ (μg/L ferritin) + 642 (r = 0.98). The lowest detectable dose was 0.8 μg/L. The within-assay precision was 7.4% based on 50 serum samples assayed in duplicate (geometric mean concentration 41 μg/L). The between-run standard deviation for 10 consecutive runs was 5, 5, and 7 μg/L for sera containing 9, 35, and 55 μg/L, respectively. Standard ferritin diluted in buffer, either containing 4 g/L bovine serum albumin or 5% (vol/vol) normal human serum, resulted in parallel standard curves.

Serum ferritin binding to concanavalin A-Sepharose

Serum (1 mL) was diluted with 1 mL 0.05 mol/L sodium acetate, pH 4.8 and heated to 80°C for 10 min. After rapid cooling on ice, the precipitate was removed by centrifugation at 3000 × g for 15 min. The pH of the supernatant was adjusted to pH 6.8 with 0.1 mol/L sodium hydroxide. Concanavalin A-Sepharose (8 mg concanavalin A/mL Sepharose) was washed on a glass filter with 0.05 mol/L Veronal, pH 8.0, 0.5 mol/L sodium chloride, 3 mmol/L sodium azide (buffer A). The Sepharose was resuspended in twice its packed volume in buffer A. Heat-treated serum (0.2 mL) was mixed with up to 1 mL concanavalin A-Sepharose and the final volume was adjusted to 2.2 mL with buffer A. After 2 hours incubation at room temperature under gentle shaking, the suspension was centrifuged at 2000 × g for 15 min. One mL supernatant was collected and assayed for unbound ferritin with the sensitive radioimmunoassay, described hereafter. In some experiments, the specificity of the binding to concanavalin A-Sepharose was tested by adding alpha-D-methylglucoside to a final concentration of 50 mmol/L together with the ferritin sample. Neither heat-treatment nor the presence of 50 mmol/L alpha-D-methylglucoside affected the serum ferritin content (unpublished observations).

Sensitive radioimmunoassay

Sera, which were absorbed with concanavalin A-Sepharose up to four times, contained insufficient ferritin to assay with our standard assay. Therefore, the sera were extracted three times according to the following method, using the same reagents as for the conventional radioimmunoassay. Antiserum (1 mL) diluted 10,000-fold was placed in plastic tubes of 1 × 7 cm and rotated for 30 min under an angle of 30 degrees (16). The tubes were washed once with 2 mL 0.05 mol/L sodium veronal (pH 8.0), 0.077 mol/L sodium chloride, 1.5 mmol/L sodium azide, 1 g/L bovine serum albumin (buffer B) and once with 2 mL distilled water. The ferritin sample (200 μL) was incubated for 60 min under rotation as described for the antiserum coating, and washed once with buffer B. This incubation was repeated twice, so that ferritin was extracted from 600 μL sample in total. Radiolabelled antiferritin in buffer B (200 μL) containing 70,000 cpm was added next and

TABLE 1
Laboratory Test Results† in Patients Suspected of Iron Deficiency

	Men	Older women	Younger women	Children
Serum ferritin, μg/L (men: 15–150; women: 7–150)*	46 (10–400)	25 (0–102)	14 (0–106)	15 (7–29)
Hemoglobin, mmol/L (men: 8.7–11.2; women: 7.5–9.9)*	9.1±0.9 (6.7–10.5)	8.3±0.7 (7.2–9.1)	8.5±0.6 (6.8–9.4)	8.2±0.7 (7.6–9.3)
Serum iron, μmol/L (14–32)*	15.3±7.5 (4.2–33.8)	13.3±6.0 (3.5–24.6)	18.8±7.7 (5.6–36.6)	17.3±7.6 (10.1–29.5)
Total iron binding capacity, μmol/L (54–72)*	56.0±8.2 (37.2–69.6)	54.1±11.7 (35.4–78.9)	64.6±10.3 (48.6–91.2)	62.4±7.4 (54.6–72.0)
Transferrin saturation (0.25–0.50)*	0.29±0.19 (0.06–0.91)	0.26±0.14 (0.07–0.57)	0.31±0.13 (0.08–0.65)	0.29±0.14 (0.14–0.51)

†Results are expressed as geometric mean with range in brackets for serum ferritin, and as mean ± S.D. with range in brackets for the other tests.
*Reference range.

TABLE 2
Serum Ferritin in Healthy Subjects and in Patients with Iron Storage Disorders

		Serum ferritin (μg/L)	
Subjects*	*n*	Geometric Mean	Range
Men	56	44	7–158
Women (premenopausal)	33	16	4–56
Women (postmenopausal)	14	36	14–126
Anemic females	16	9	2–77
Iron overload	10	1473	379–2814

*See Patients and Control Subjects section for description.

incubated under rotation as described for the antiserum coating. The tubes were washed once with buffer B and the tube-associated radioactivity was counted. With this assay, a linear standard curve (r = 0.99) from 0.2 to 6 μg/L ferritin was obtained with a slope of 1421 cpm/μg/L ferritin and a blank value of 264 cpm.

OTHER METHODS

Ferritin protein was measured according to Schacterle and Pollack (17). Ferritin iron was measured with bathophenantroline according to Harris (18). Hemoglobin was measured with the Coulter Counter S (Coulter Counter Electronics, Inc., Hialeah, FL, USA). Total iron in serum was measured with ferrozine chromogen on an autoanalyzer system (Technicon Instr. Corp. Tarrytown, NY, USA, method SG4-0025 FH9) with Technicon reagents. Total iron binding capacity (TIBC) was measured on the same system with magnesium carbonate after saturation with ferric chloride (reagents from J. T. Baker, Deventer, The Netherlands). The transferrin saturation was calculated by dividing the serum iron by the TIBC. Radioactivity was measured with a gamma counter (Trigamma 600; Baird Atomic Inc., Bedford, MA, USA).

Patients and control subjects

Control subjects were ostensibly healthy subjects taken at random from the hospital staff. The mean age of the male group was 27 years (range 22–57, n = 56). The female group was divided into a premenopausal group (mean age 28 years, range 22–45, n = 33), and a postmenopausal group (mean age 57 years, range 50–63, n = 14).

Anemic patients were women (mean age 24 years, range 18–36, n = 16) 7 days after delivery, who had hemoglobin levels less than 7.2 mmol/L (6.5 ± 0.5 mmol/L, mean ± SD, range 5.4–7.1).

Sera from ambulant patients suspected of iron deficiency were obtained from a clinical laboratory serving general practitioners in the Utrecht area (The Netherlands). This group consisted of 16 males (mean age 42 years, range 14–77), 50 younger females (mean age 26 years, range 13–43), 15 older females (mean age 61 years, range 46–87), and 5 children (mean age 8 years, range 3–9). The relevant laboratory test results are given in Table 1.

Sera of 43 patients with a wide range of serum ferritin concentrations were obtained by collecting, during one month, all sera sent to our laboratory for routine serum ferritin measurements. These latter sera were used only for comparison of our ferritin assay method with a commercially available ferritin RIA (The Radiochemical Centre).

Results

SERUM FERRITIN CONCENTRATIONS IN HEALTHY SUBJECTS AND PATIENTS

Serum ferritin concentrations in control subjects and in patients with iron storage disorders are shown in Table 2. The data were skewed in the upward direction and, therefore, the data are presented as geometric mean and range. In post-menopausal women, higher serum ferritin concentrations were measured than in

TABLE 3
Decreased Hemoglobin, Serum Iron, and Transferrin Saturation in Patients with Different Serum Ferritin Levels

Serum ferritin group (μg/L)	Total number of patients	Number of patients with decreased		
		Hemoglobin	Serum iron	Transferrin saturation*
1–4	6	1	6	5
5–15	25	4	6	3
16–25	23	3	8	3
26–400	31	2	10	4

Patients suspected of iron deficiency, described in the Patients and Control Subjects section, were grouped according to their serum ferritin level. The number of patients with abnormal results for the other iron parameters indicating iron deficiency were recorded. The complete laboratory test results and reference values are listed in Table 1.
*Transferrin saturation below 0.16.

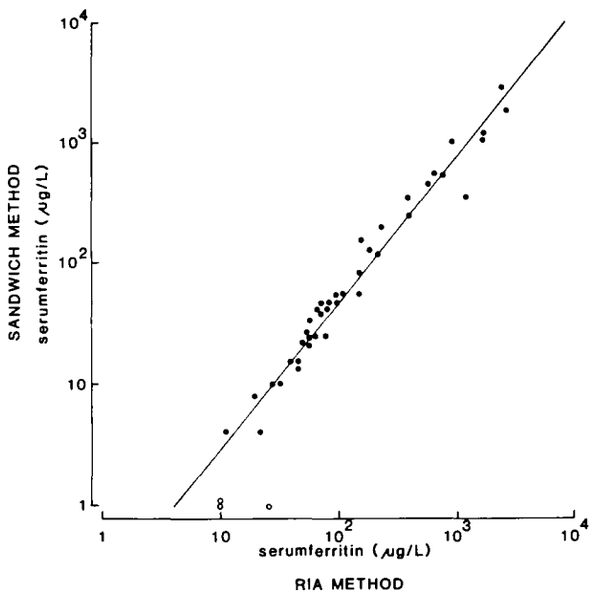

Figure 1 — Correlation of our assay (sandwich method) with the commercial radioimmunoassay kit for serum ferritin from The Radiochemical Centre (RIA method). 43 patient samples were assayed by both methods. The RIA method was carried out according to the instructions of the manufacturer. (○): samples which were not included in the regression analysis.

younger women. In fact, the distribution of the older females was close to that of the male control group, while the distribution of the younger women was very close to that of anemic women. In patients with excess liver iron, serum ferritin concentrations were one to two orders of magnitude higher than in the control subjects.

CORRELATION WITH COMMERCIALLY AVAILABLE RADIOIMMUNOASSAY FOR SERUM FERRITIN

Sera from 43 patients selected at random were assayed both with our method (sandwich method), as well as with the radioimmunoassay kit from The Radiochemical Centre (RIA method). There was a substantial difference between the two methods for ferritin-poor sera. Three sera which had serum ferritin concentrations <1 μg/L in the sandwich method, gave by the RIA method levels of 10, 10, and 27 μg/L. For the remaining sera having ferritin concentrations in the normal range or higher, a good correlation was observed between the two methods (Figure 1). The equation of the regression was:

$$\log y(\text{sandwich method}) = 1.19 \log x(\text{RIA method}) - 0.68;\ r^2 = 0.97,\ p < 0.001$$

COMPARISON OF SERUM FERRITIN WITH OTHER INDICATORS OF IRON-DEFICIENCY

Serum ferritin, hemoglobin, serum iron, transferrin saturation, and total iron-binding capacity were measured in sera of ambulant patients suspected of iron deficiency. In six patients with very low serum ferritin concentrations (less than 5 μg/L), all six had reduced serum iron, five out of six reduced transferrin saturation, but only one patient had also a reduced hemoglobin level. In 25 patients with subnormal serum ferritin concentrations (5–15 μg/L) only four, six, and three patients respectively had decreased hemoglobin, serum iron, or transferrin saturation. In patients with normal serum ferritin concentrations or higher, the incidence of decreased hemoglobin, serum iron, and transferrin saturation was about the same as seen in the patient group with subnormal serum ferritin concentrations (Table 3). Serum ferritin showed an inverse relationship with the total iron binding capacity (Figure 2, Spearman rank correlation coefficient: 0.56; $p < 0.01$). No significant correlation was observed for serum ferritin and any of the other iron parameters.

BINDING OF SERUM FERRITIN TO CONCANAVALIN A

When normal serum was incubated with concanavalin A-Sepharose, 70% of the serum ferritin bound to the immobilized concanavalin A (Figure 3). The remaining ferritin lacked concanavalin A-binding properties, as there was no difference in binding whether 0.5 or 1 mL concanavalin A-Sepharose was

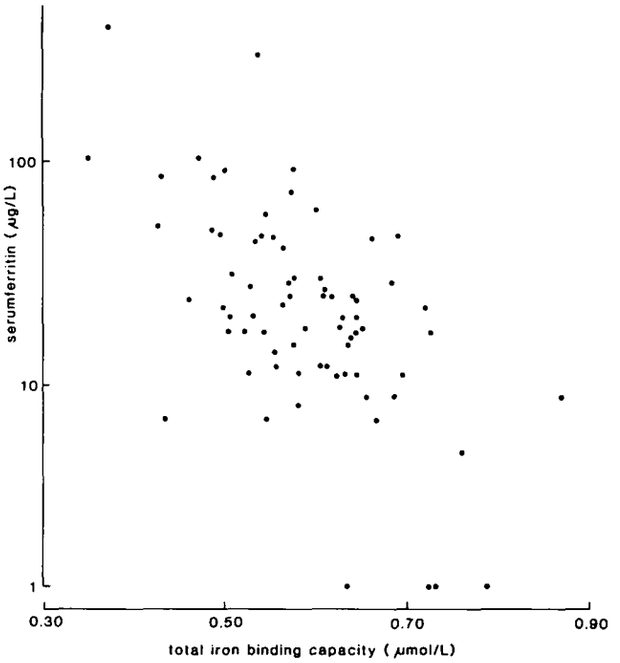

Figure 2 — Relation between serum ferritin and total iron binding capacity in 73 patients suspected of iron deficiency. Patients using birth control pills were excluded from the analysis.

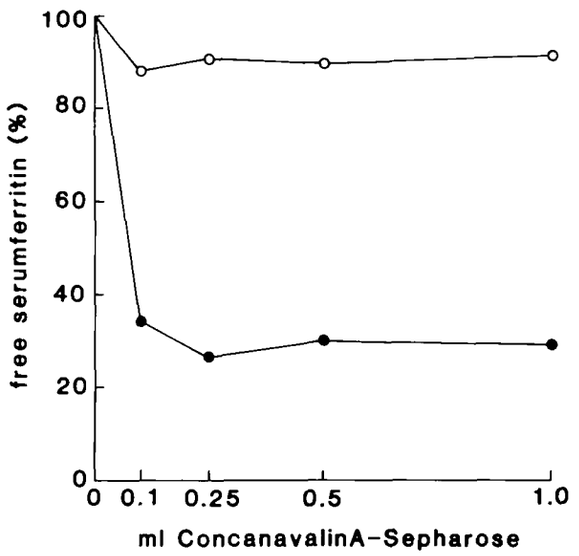

Figure 3 — Serum ferritin binding to concanavalin A-Sepharose. The ferritin fraction in heat-treated serum which did not bind to the indicated amounts of concanavalin A-Sepharose was assayed as described in the Materials and Methods section and expressed as percent of the total amount of ferritin present in the mixtures. The heat-treated serum contained 44 µg/L ferritin. (●): serum ferritin binding to concanavalin A-Sepharose; (○): serum ferritin binding to concanavalin A-Sepharose in the presence of 50 mmol/L alpha-D-methylglucoside.

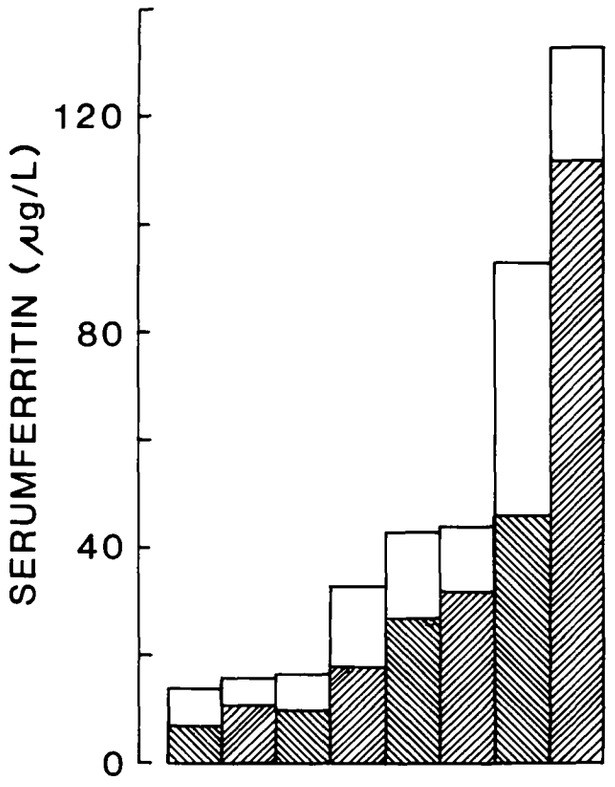

Figure 4 — Binding of serum ferritin to concanavalin A-Sepharose in normal subjects. Eight sera from healthy subjects were heat-treated and the fraction of serum ferritin which did not bind to concanavalin A-Sepharose was assayed as described in the legend of Figure 3. The total serum ferritin concentration is indicated by the vertical bars and the fraction of ferritin which did bind to concanavalin A-Sepharose is indicated by the shaded areas.

added. In the presence of 50 mmol/L alpha-D-methylglucoside, an inhibitor of the binding of glycoproteins to concanavalin A, only 10% binding was observed.

The fraction of serum ferritin which bound to concanavalin A-Sepharose in 8 normal sera is shown in Figure 4. With increasing serum ferritin concentration, an increasing amount of serum ferritin bound to concanavalin A-Sepharose.

Discussion

The serum ferritin concentrations we found in healthy subjects, in iron-deficient anemia patients, and in iron-overload patients are in accordance with previous reports (2, 3). However, our actual data are somewhat lower than reported in the literature. This is most probably due to the use of different standards (9). The separation between patients and healthy control subjects was better for the iron-overload patients than for the iron-deficient anemia patients, which is in harmony with the prevalence of these disorders in the population (19–21). In contrast to men and postmenopausal women, serum ferritin concentrations in younger women were found to be close to those in iron-deficient anemia patients. This is due to the fact that pre-

menopausal women have little or no storage iron (22, 23), but apparently just enough to maintain adequate erythropoiesis (24).

To compare our method with a commercial serum ferritin radioimmunoassay, results were obtained from subjects with a wide range of serum ferritin concentrations. Our sandwich method compared reasonably well with the competitive ferritin radioimmunoassay method for sera with normal or elevated ferritin concentrations. However, absolute values differed for the two methods which is, as mentioned before, probably due to lack of standardization. The remarkable high discrepancy for ferritin-poor sera cannot be explained at present. It may be due to the greater contribution of the a-specific binding to the total binding in radioimmunoassay procedures *versus* sandwich methods.

In a group of patients suspected of iron deficiency by general practitioners, serum ferritin concentrations ranged from 1–400 μg/L. In patients with very low serum ferritin concentrations (<5 μg/L), all had reduced serum iron and the transferrin saturation was below 0.16 in 5 out of 6 patients. A transferrin saturation under 0.16 is considered to be a reliable indicator for iron deficiency, provided anemia of chronic disease is excluded (25). Jacobs *et al.* (26) reported that serum ferritin concentrations below 10 μg/L were associated with transferrin saturation under 0.16. The difference in nominal ferritin values is probably due to lack of standardization because our normal values were about half of those presented by Jacobs *et al.* (26). Also, Mazza *et al.* (27) reported that, in patients selected for absent bone marrow iron stores, serum ferritin and transferrin saturation had about the same sensitivity for detection of iron deficiency. In patients with subnormal ferritin concentrations (5–15 μg/L) the incidence of reduced serum iron and transferrin saturation was much lower, compatible with latent iron deficiency in this group. It was suggested before that the ferritin assay may detect iron deficiency in patients with otherwise normal iron parameters (28). Also, in phlebotomized normals, serum ferritin was a more sensitive indicator for iron deficiency (29, 30). Serum ferritin was found to be inversely correlated with the total iron binding capacity, which was also found by other investigators (31, 32). The fact that serum ferritin is also an acute phase protein (33) complicates its use for diagnosis of mild iron overload. On the other hand, serum ferritin remains very useful to distinguish between anemia due to iron deficiency and anemia due to other causes, e.g. anemia of chronic disease.

Plasma contains numerous proteins which bind to concanavalin A. A large fraction of serum ferritin has also been shown to bind specifically to concanavalin A. Heat-treatment of serum reduces the amount of concanavalin A-Sepharose required by 75%, without affecting the binding itself. A small fraction of serum ferritin binds in a non-specific manner, as it could not be displaced by alpha-D-methylglucoside. This reflects probably a hydrophobic component in the interaction of concanavalin A with its ligands (34). Evidently a fraction of serum ferritin did not bind to concanavalin A. This fraction of serum ferritin might be directly related to ferritin in tissues because ferritin isolated from human liver and heart tissue does not bind to concanavalin A (van Oost, unpublished experiments). In the normal subjects, the amount of serum ferritin which bound to concanavalin A was proportional to the total amount of serum ferritin in the sample. This indicates that in normal subjects the levels of glycosylated and nonglycosylated serum ferritin are regulated by the same mechanisms.

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