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Detection of a glycosylated subunit in human serum ferritin

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Ferritin was purified from the serum of two patients with idiopathic haemochromatosis. The protein contained three types of subunit—the H and L subunits of tissue ferritins (although only a trace of H could be detected) and a third subunit, 'G', with the highest apparent molecular weight. Only the 'G' subunit band stained for carbohydrate, indicating that a proportion of the subunits of human serum ferritin are glycosylated. Although serum was obtained from patients with idiopathic haemochromatosis, it is probable that the 'G' subunit is a component of normal serum ferritin.

The iron storage protein, ferritin, is found in all tissues. It contains 24 subunits (Harrison *et al.*, 1974) of two types, H and L (Arosio *et al.*, 1978) and molecules comprising different proportions of H and L subunits are thought to form the various isoferritins seen on isoelectric focusing. Low concentrations of ferritin are found in normal plasma and are known to be closely related to the amount of storage iron in the body (Worwood, 1980*a*). A high proportion of normal serum ferritin binds to concanavalin A, a plant lectin that binds glycoproteins containing mannose and/or glucose residues, suggesting that the protein is glycosylated (Worwood *et al.*, 1979). Much of the ferritin from the serum of patients with idiopathic haemochromatosis binds to concanavalin A, but when there is extensive liver damage liver ferritin is released into the plasma and the proportion that binds to concanavalin A is decreased (Worwood *et al.*, 1979). Further evidence for glycosylation was provided by incubating partially purified serum ferritin, obtained from patients with idiopathic haemochromatosis, with neuraminidase. Serum ferritin showed a wide range of isoferritins on isoelectric focusing, but, after treatment with neuraminidase, the more acidic isoferritins disappeared, presumably due to the removal of sialic acid residues (Cragg *et al.*, 1980). This indicates that much of the microheterogeneity of serum ferritin is due to glycosylation rather than to variation in the proportions of H and L subunits. In this paper, we provide evidence that serum ferritin contains a glycosylated subunit. Ferritin has been purified from serum obtained from patients with idiopathic haemochromatosis undergoing removal of iron by phlebotomy. These patients provide the only convenient source of the large amount of serum of high ferritin concentration required for purification.

Abbreviation used: SDS, sodium dodecyl sulphate.

Experimental

Purification of serum ferritin

Serum (preparation I) or plasma (preparation II) was obtained from two patients with idiopathic haemochromatosis. Batches of between 500 and 1220 ml of plasma or serum were processed by diluting with an equal volume of 0.05 M-sodium acetate solution, adjusting the pH to 4.8 with 1 M-acetic acid, heating rapidly to 70°C and then maintaining this temperature for 10 min, before cooling in ice and centrifuging at 10000 ***g*** for 30 min. The supernatant was filtered, the pH adjusted to 6.8 with 1 M-NaOH and the extract concentrated to approx. one-fifth of its volume by ultrafiltration (Amicon XM 100A membrane). Each extract was then subjected to affinity chromatography with antibodies to human spleen ferritin (Worwood *et al.*, 1976) and the eluates from four batches of serum from the same patient were combined and concentrated by ultrafiltration (Amicon XM 100A membrane). Chromatography on Sepharose 6B was followed by ion-exchange chromatography on Sephadex A-50 and gel filtration on Sephadex G-200 (Worwood *et al.*, 1976).

Purification of tissue ferritins

Ferritin was purified from iron-loaded spleen obtained at operation and from iron-loaded heart obtained *post mortem*, by using the method of Worwood *et al.* (1975). Human liver ferritin was purified by a procedure including repeated crystallization from cadmium sulphate as described by Ryan *et al.* (1978).

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Polyacrylamide-gel electrophoresis

Electrophoresis was carried out at pH 7.5 using 4% (w/v) polyacrylamide gels (Worwood *et al.*, 1976), cross linked ($C = 15\%$) with *N,N'*-diallyltartardiamide (Görg *et al.*, 1978). Power was applied to the gels (70 mm × 70 mm × 1 mm) for 3 h at 1.5 W per plate at 4°C. Samples (7 μl) were applied, containing 2 μg of protein for protein stain, 4 μg of protein for iron stain or 0.5 μg of protein for ferritin precipitation with antiserum to purified human spleen ferritin. Fixing, staining and immunoprecipitation were carried out as described by Worwood *et al.* (1976).

Isoelectric focusing

Isoelectric focusing in flat beds of 5% (w/v) polyacrylamide gel crosslinked ($C = 15\%$) with *N,N'*-diallyltartardiamide was carried out according to the method of Wagstaff (1980).

SDS gradient-pore polyacrylamide-gel electrophoresis

Subunit analysis was performed on purified ferritin samples according to the method of O'Farrell (1975) as modified by Wagstaff (1980). Molecular weight marker proteins (Sigma; Dalton Mark VI) and protein controls for carbohydrate staining were also subjected to electrophoresis in SDS. Samples of 2 μg were applied for protein staining and 20 μg samples were applied to gels for staining for carbohydrate. Stained gels were scanned with a Pye SP 8100 spectrophotometer fitted with a densitometer attachment (Pye Unicam). In some cases gels were loaded with 20 μg of serum ferritin and after electrophoresis the protein bands were visualized in 1 M-KCl (Arosio *et al.*, 1978) before being cut from the gels with a scalpel blade. The bands were separately eluted overnight into 0.025 M-Tris buffer containing 0.192 M-glycine, 0.1% SDS and 0.01 M-mercaptoethanol, and the eluates were then dialysed for 2 weeks against many changes of 0.05 M-sodium barbitone buffer, pH 8.0, containing 0.1 M-NaCl, 0.02% NaN_3, 5 g of bovine serum albumin/l and 0.01 M-mercaptoethanol. The preparations from the subunit bands were finally assayed for ferritin by using the immunoradiometric assay (see under 'Other methods').

Concanavalin A binding

Determination of the percentage of binding to concanavalin A by ferritin in serum and by purified serum ferritin preparations was carried out by the method of Worwood *et al.* (1979). An aliquot of each purified serum ferritin preparation was also separated into its concanavalin A binding and non-binding fractions (Worwood *et al.*, 1979) by passing the solution of purified ferritin through a 1 ml column of concanavalin A–Sepharose 4B (Pharmacia) and eluting ferritin bound to the column with 0.05 M-α-D-methyl glucopyranoside.

Carbohydrate detection

Staining for carbohydrate in SDS/polyacrylamide gels was carried out by using the thymol/H_2SO_4 method of Racusen (1979). Binding to concanavalin A was investigated by the method of Furlan *et al.* (1979) using concanavalin A conjugated to fluorescein isothiocyanate.

Crossed immunoelectrophoresis

Electrophoresis of 1 μg of serum ferritin was carried out in polyacrylamide gels (see above) and a strip containing the protein was removed and transferred to an 80 mm × 80 mm glass plate. After embedding the strip and covering the plate in 10 ml of agarose (0.8% in $I = 0.05$ barbital/acetate buffer, pH 8.6, containing the optimum amount of affinity-purified sheep antibodies to human ferritin) electrophoresis was carried out in the second dimension at 200 V for 1 h followed by 120 V for 18 h. The plates were stained with Coomassie Blue after drying.

Iron uptake

Iron uptake studies were carried out according to the method of Wagstaff *et al.* (1978). Iron (1000 atoms/molecule) was added to 52.5 μg of ferritin in 1 ml of $I = 0.05$ barbitone/HCl buffer, pH 7.0; both serum ferritin that bound to concanavalin A and serum ferritin that did not bind to concanavalin A were studied in this way. In a separate experiment, iron (500 atoms/molecule) was added to native serum ferritin and to serum ferritin from which the iron had been removed by reduction with sodium dithionite (Macara *et al.*, 1972). The preparations were then subjected to isoelectric focusing.

Other methods

Protein was measured by the method of Lowry *et al.* (1951) using bovine serum albumin as standard. Ferritin concentrations were determined by immunoradiometric assay with spleen ferritin standards and anti-(spleen ferritin) antibodies (Jones & Worwood, 1978). Antibodies to purified human spleen ferritin were raised in rabbits and sheep as described by Worwood (1980*b*). Specificity was tested by immunoelectrophoresis against spleen ferritin and human serum. Antibodies to human spleen ferritin raised in sheep were purified by passing sheep antiserum through an immunoadsorbent column of 'crystalline' liver ferritin coupled to CNBr-activated Sepharose 4B (Pharmacia) and eluting the antibody with 3 M-$MgCl_2$ in 0.01 M-phosphate buffer, pH 7.6. Iron was estimated by flameless atomic absorption spectrophotometry (Varian models 1100 and 63).

Results

Table 1 summarizes the purification of ferritin from the two patients. Overall recovery of serum ferritin was 16% in preparation I and 23% in preparation II, and both preparations after gel filtration on Sephadex G-200 apparently contained 78% ferritin, as determined from the ratio of ferritin concentration measured by immunoradiometric assay to protein concentration measured by the Lowry method. This represented purifications of 15500-fold and 28500-fold in preparations I and II respectively. The iron content of both preparations was low. Iron:protein ratios measured as μg of iron per μg of protein were 0.038 for preparation I and 0.022 for preparation II. The percentage of ferritin binding to concanavalin A was identical in both the original serum and in the purified material (preparation I, 55%; preparation II, 42%).

On polyacrylamide-gel electrophoresis both preparations of serum ferritin gave two bands that reacted with rabbit anti-(spleen ferritin) antibody (Fig. 1*a*). Both bands gave peaks with a continuous line of precipitation, indicating immunological identity, when subjected to crossed immunoelectrophoresis using sheep anti-(spleen ferritin) antibodies

Table 1. *Purification of serum ferritin*
Abbreviation: n.a., not available

	Stage	Total ferritin (μg)	Total protein (mg)	Recovery of ferritin (%)	Specific concn. of ferritin	Binding to concanavalin A (%)
Serum ferritin	Serum (2110ml)	6800	134900		0.00005	55
Preparation I	Heat-treatment	5438	2820	80	0.0019	
	Affinity chromatography	4753	n.a.	70	n.a.	
	Sepharose 6B gel filtration	2605	5.45	38	0.48	
	Sephadex A-50 chromatography	2102	2.77	31	0.76	
	Sephadex G-200 gel filtration	1084	1.39	16	0.78	55
Serum ferritin	Serum (3190ml)	6920	252700		0.00003	42
Preparation II	Heat treatment	5674	5200	82	0.0011	
	Affinity chromatography	3525	11.31	51	0.31	
	Sepharose 6B gel filtration	2737	3.25	40	0.84	
	Sephadex A-50 chromatography	2557	3.19	37	0.80	
	Sephadex G-200 gel filtration	1614	2.07	23	0.78	42

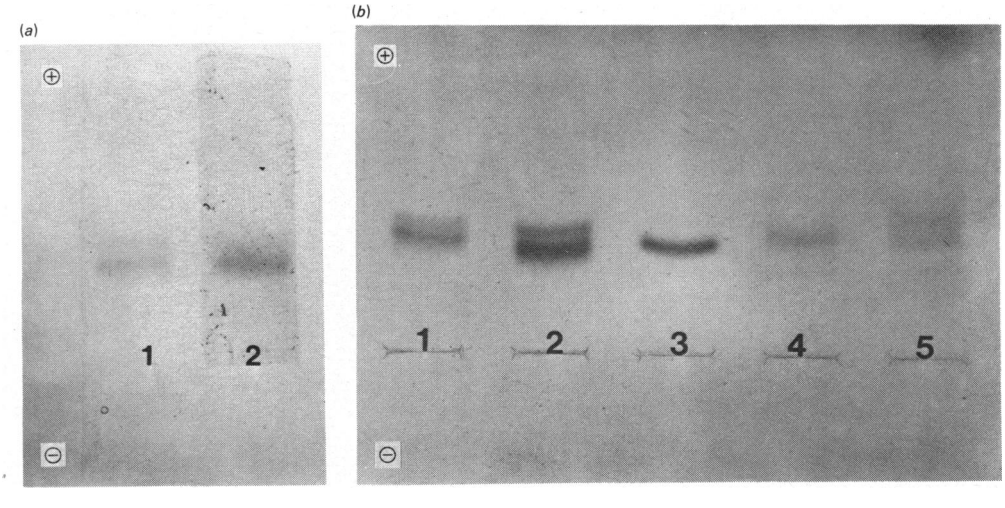

Fig. 1. *Polyacrylamide-gel electrophoresis of human ferritin*
(*a*): (1) Serum ferritin stained directly with Coomassie Blue; (2) serum ferritin stained with Coomassie Blue after immunofixation with rabbit anti-(spleen ferritin) antibody. (*b*): (1) Whole serum ferritin; (2) serum ferritin that bound to concanavalin A; (3) serum ferritin that did not bind to concanavalin A; (4) spleen ferritin; (5) heart ferritin. The gel was stained with Coomassie Blue.

that had been purified using an immunoadsorbent of 'crystalline' liver ferritin. The fraction of serum ferritin that bound to concanavalin A and was then eluted also showed two bands on polyacrylamide-gel electrophoresis, but the non-binding fraction contained only one band corresponding to the slower-moving of the two serum ferritin bands (Fig. 1*b*). Only the band of lower mobility from purified serum ferritin and the band obtained from the non-binding fraction of serum ferritin stained for iron.

Isoelectric focusing of the serum ferritin preparations showed the presence of a large number of isoferritin bands covering a wide range of pI values from the most basic isoferritins of spleen ferritin to the most acidic bands of heart ferritin (Fig. 2*a*). The fraction of serum ferritin that did not bind to concanavalin A contained only the most basic isoferritins, whereas the ferritin that bound to concanavalin A contained a wide range of isoferritins. All the bands that stained for protein also stained for protein after immunofixation with rabbit anti-(spleen ferritin) antibodies in unfixed gels (Fig. 2*b*).

Three bands were demonstrated in purified serum ferritin subjected to SDS-gradient pore electrophoresis (Fig. 3). Two of these corresponded to the

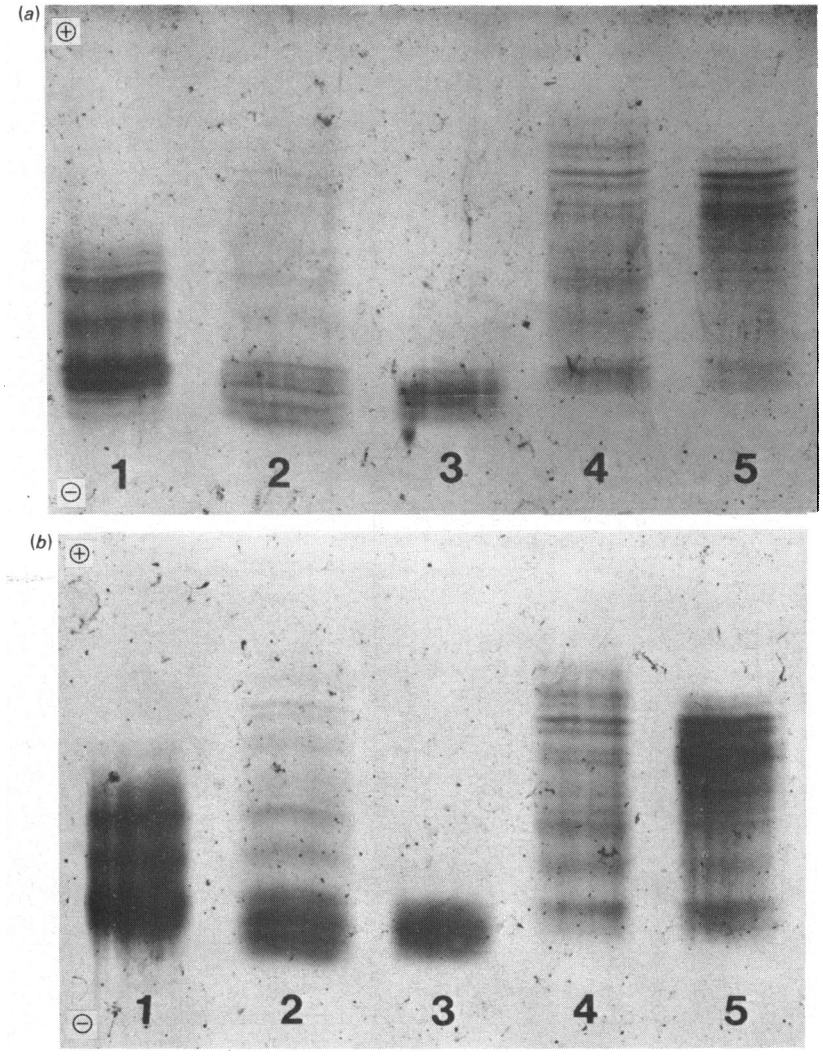

Fig. 2. *Isoelectric focusing of human ferritin*
(*a*) Gel stained directly with Coomassie Blue. (*b*) Gel stained with Coomassie Blue after immunofixation with rabbit anti-(spleen ferritin) antibody. (1) Spleen ferritin; (2) whole serum ferritin; (3) serum ferritin that did not bind to concanavalin A; (4) serum ferritin that bound to concanavalin A; (5) heart ferritin.

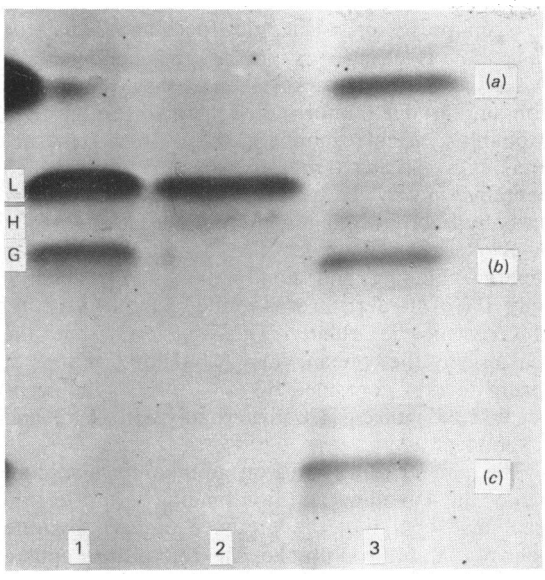

Fig. 3. *SDS-gradient pore electrophoresis*
(1) Whole serum ferritin; (2) spleen ferritin; (3) molecular weight marker proteins: (*a*) lysozyme (mol.wt. 14300); (*b*) trypsinogen (24000); (*c*) egg albumin (45000).

H and L subunit bands of spleen and heart ferritins, although only a trace of the H subunit could be detected. There was also a third band (which we have called 'G') with mol.wt. approx. 23000. Densitometric scanning showed that the 'G' band accounted for 27% and 24% of serum ferritin in preparations I and II respectively. A similar analysis of the separated concanavalin A binding and non-binding fractions showed that the latter fractions from both preparations contained 100% L type subunit. The concanavalin A binding fractions were enriched with protein in the 'G' band, preparation I containing 47% and preparation II 32% of total protein in this band.

SDS/polyacrylamide gels which were stained for carbohydrate included tracks with bovine serum albumin and transferrin as negative and positive controls respectively. Albumin, spleen and heart ferritins did not stain for carbohydrate. Transferrin and one band of each serum ferritin preparation stained for carbohydrate (thymol/H_2SO_4 reaction) and fluoresced after incubation with fluorescein-conjugated concanavalin A. The carbohydrate staining band of serum ferritin was marked by slitting the gel with a scalpel. After staining the same gel for protein, the slit band was shown to correspond to the 'G' band.

Elution and dialysis of the L band from SDS/polyacrylamide gels resulted in a preparation that assayed as ferritin and represented 38% of the total ferritin applied to the gel. The material assaying as ferritin was eluted at the void volume of a Sephadex G-200 column, showing that the subunits had aggregated to form apoferritin molecules. The preparation from the 'G' band showed only a low level of cross-reactivity with anti-(spleen ferritin) antibodies in the assay (approx. 10% of the expected ferritin concentration) and the dilution curve was not parallel with those for spleen ferritin. The low levels of ferritin detected by immunoradiometric assay made it impossible to examine the elution volume on Sephadex G-200.

Iron uptake curves for both concanavalin A binding and non-binding serum ferritin were similar to those obtained with human liver and spleen ferritins, except that the rate of uptake was markedly slower by the serum ferritin components ($t_{\frac{1}{2}}$ bound 18.5 min, $t_{\frac{1}{2}}$ unbound 19.5 min, $t_{\frac{1}{2}}$ human liver ferritin, 5.9 min, mean of six samples). The measurements of rate of iron uptake by serum and liver ferritins were carried out under the same conditions but not at the same time.

Only the most basic isoferritins of native serum ferritin were found to contain iron after staining gels by the Prussian Blue reaction but, after the addition of iron to both native serum ferritin and serum apoferritin produced by dithionite reduction, iron was seen to have been incorporated into the full range of isoferritins. The isoelectric focusing patterns after staining for protein were identical before and after addition of iron, indicating that the iron was incorporated inside the apoferritin shell because there was no change in surface charge.

Discussion

The combination of heat treatment and affinity chromatography has made it possible to obtain reasonable yields of highly purified serum ferritin without selection of either the ferritin that does or does not bind to concanavalin A. The usual test for purity of ferritin is the demonstration that the bands seen on polyacrylamide-gel electrophoresis in non-dissociating buffer stain for both iron and protein and correspond to the monomers and polymers of ferritin. This test cannot be applied to serum ferritin, which contains very little iron. Instead, evidence of purity must rely on immunological methods, on molecular weight and on the ability to take up iron. Both polyacrylamide-gel electrophoresis and isoelectric focusing indicated that the two serum ferritin preparations were pure, since the bands that stained directly for protein also stained for protein after immunofixation with specific antisera to human spleen ferritin. The immunological identity of the two bands seen on polyacrylamide-gel electrophoresis

was demonstrated by crossed immunoelectrophoresis. Additional evidence of purity was obtained by demonstrating iron uptake by both the more basic and the acidic serum isoferritins. Chromatography on Sepharose 6B demonstrated identical elution volumes for spleen and serum ferritins, with no evidence of additional peaks or shoulders in the serum ferritin peak. In both preparations the ferritin content was 0.78 μg of ferritin/μg of protein, as determined by comparison between the immunoradiometric assay of ferritin and the protein content determined by the Lowry method. This value depends on the immunoreactivity of the ferritin employed as a standard in the immunoassay. Four other preparations of spleen ferritin showed values of 0.72–0.87 μg of ferritin/μg of protein (mean 0.80) with the same standard (Wagstaff, 1980).

It is necessary to establish the purity of the serum ferritin preparations before considering the bands seen after gradient gel electrophoresis in SDS. Two of the three bands seen corresponded to the H and L subunits of human tissue ferritins, which have mol.wts. 21000 and 19000 respectively (Arosio *et al.*, 1978). The third subunit, 'G', was of apparent mol.wt. 23000 and was the only band staining for carbohydrate.

The L subunit band of serum ferritin could be eluted from the gel and reassembled into apoferritin molecules. This was shown by its binding to antibodies to spleen ferritin in the immunoradiometric assay and its elution at the void volume during chromatography on Sephadex G-200. The dilution curve for this ferritin assembled from L subunits was parallel to that for the standard spleen ferritin. However, the material in the 'G' band, although showing binding in the immunoradiometric assay, did not dilute in parallel with spleen ferritin. The apparent ferritin concentration was only about 10% of the expected concentration (calculated from the amount of L subunit eluted from the same gel). It is possible that steric hindrance prevents the formation of apoferritin from 'G' subunits alone and it is not known how subunits would react with antibodies to ferritin in the immunoradiometric assay. Serum ferritin from patients with iron overload shows no binding to anti-(heart ferritin) antibodies (Jones & Worwood, 1978), showing that the 'G' subunit is likely to be a derivative of the L subunit rather than of the H subunit. Furthermore, incubation of serum ferritin with neuraminidase converts serum ferritin to molecules of pI 5.7 (Cragg *et al.*, 1980), very similar to that of the homopolymer of L subunits (Arosio *et al.*, 1977).

Serum ferritin is heterogeneous in terms of carbohydrate content. The 'non-binding' fraction appears to contain no carbohydrate and shows very limited microheterogeneity on polyacrylamide-gel electrophoresis or isoelectric focusing. The wide range of isoelectric points of the concanavalin A-binding ferritin suggests that these molecules contain variable numbers of sialic acid residues and probably variable numbers of glycosylated subunits. On passing 1 mg of spleen ferritin (which contains mostly L subunits and less than 0.5% carbohydrate) through a column of concanavalin A–Sepharose 4B under the conditions used to separate binding and non-binding serum ferritin, only 1% of the ferritin was found in the α-D-methyl glucopyranoside eluate. This suggests that the reason why the concanavalin A-binding fractions of serum ferritin contained 53–68% of L subunits is because the molecules are heteropolymers of 'G' and L subunits.

The similar rates of iron uptake by the concanavalin A-binding and non-binding serum ferritin fractions show that the presence of carbohydrate does not inhibit iron uptake. The rate of iron uptake by serum ferritin ($t_{\frac{1}{2}}$ 19 min) is comparatively slow but approaches the rate ($t_{\frac{1}{2}}$ 14 min) found for a fraction of liver ferritin prepared by ion-exchange chromatography which contained only L subunits (Wagstaff, 1980).

Our results confirm that the microheterogeneity of serum ferritin is largely due to glycosylation rather than to variation in the proportion of H and L subunits. As the carbohydrate is present after purification of ferritin and dissociation into subunits it must be covalently bound to the peptide chain. It seems unlikely that non-enzymic glycosylation of ferritin occurs in the plasma, firstly because animal studies show that the half-time for the clearance of ferritin from the plasma is of the order of 10 min (Worwood, 1980*a*), and secondly because incubation of spleen ferritin in serum for up to 48 h does not produce significant binding to concanavalin A (M. Worwood, unpublished work). We have suggested (Worwood *et al.*, 1979) that a fraction of the ferritin enters the plasma after synthesis on polysomes bound to endoplasmic reticulum (Puro & Richter, 1971), followed by glycosylation. Glycosylation of ferritin not only modifies the properties of the circulating protein but may also influence the rate of removal of ferritin from the plasma, so that glycosylated ferritin is cleared more slowly than non-glycosylated ferritin. 'G' subunits may therefore be only a small proportion of the total number of ferritin subunits synthesized. It is possible that the 'G' subunit is peculiar to patients with idiopathic haemochromatosis, but our earlier studies suggest that it is also a component of normal serum ferritin (Cragg *et al.*, 1980). Ferritin appears to be an interesting example of a protein that is largely synthesized on free polysomes for intracellular purposes but may also be secreted from the cell after synthesis on bound polysomes.

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