Properties of Human Tissue Isoferritins

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1. Human liver ferritin was separated by preparative isoelectric focusing into six fractions.
2. Except for the least acidic fraction the reactivity with antibody against spleen ferritin increased with rising pI, but with antibody against heart ferritin the reactivity decreased.
3. The highest iron content was found in the most acidic isoferritins and progressively decreased with rising pI.
4. Iron uptake was studied in apoferritin prepared from heart and liver ferritin fractions separated by ion-exchange chromatography. There was good correlation between the rate of iron uptake and pI. The most acidic fractions took up iron more rapidly than did the more basic ones.
5. Ferritin was prepared from heart, liver, spleen and kidney. There was little difference on isoelectric focusing between ferritin obtained from normal tissues and the corresponding iron-loaded tissues from patients who had received multiple blood transfusions. The iron-loaded heart ferritin invariably contained relatively more of the basic isoferritins. Normal and iron-overloaded heart ferritins were separated into isoferritin fractions by ion-exchange chromatography, and in each case there was a fall in iron content as the pI increased. The iron content of ferritin from the iron-overloaded heart was higher throughout than that from normal heart.
6. There is a relationship between the rate of iron uptake by apoferritin and pI, and this probably accounts for the variation in iron content of the isoferritins found in human liver and heart.

Ferritin is an iron-storage protein consisting of an outer protein shell of mol.wt. 450000 with a central cavity containing a variable amount of iron, deposited as a microcrystalline ferric hydroxide-phosphate complex (Harrison *et al.*, 1974). The protein component is composed of 24 subunits each of mol.wt. approx. 19000. Different tissues may contain ferritins of different charge, human heart ferritin, for example, having a lower isoelectric point than that of liver. When isoelectric-focusing techniques are used, differences between tissue ferritins are seen to be more complex than originally thought. Drysdale (1974) showed that liver ferritin was separated into about six protein bands with isoelectric points (pI) ranging from pH 5.1 to 5.6, whereas heart ferritin consisted mainly of more acidic ferritin with a pI of about 5.0 (Powell *et al.*, 1975a). The existence of multiple 'isoferritins' within a single tissue may result from molecules built up of identical subunits, each unique to a particular isoferritin, or different isoferritins built up from variable proportions of two or more subunits. The demonstration of two subunits (H and L) of different charge (Adelman *et al.*, 1975) and molecular weight (Drysdale, 1977) supports the latter hypothesis.

The reactivity of ferritin with antibody against spleen ferritin also shows variation. Heart ferritin

reacts but little (Worwood *et al.*, 1976b); liver ferritin reacts to a similar degree to spleen ferritin, and serum ferritin has a reactivity of about 50% in excess of that of spleen ferritin (Worwood *et al.*, 1976a).

In horse spleen ferritin, iron is not distributed equally between all molecules (Macara *et al.*, 1972). On ultracentrifugation a sharp apoferritin peak is separated from a broader peak of ferritin molecules with a range of iron contents. Apoferritin from horse spleen is confined mainly to the most acidic ferritins (Ishitani *et al.*, 1975), whereas in human tissues the reverse situation holds, the ferritin of lowest iron content being the most alkaline (Arioso *et al.*, 1977).

There may be changes in the isoferritin profile in patients with iron overload. Ferritins prepared from heart, pancreas and kidney of patients with untreated idiopathic haemochromatosis had apparently lost the acidic isoferritins that they normally contain and gained more basic isoferritins, resulting in an isoferritin pattern very similar to that of liver (Powell *et al.*, 1974). Similar findings were reported for Bantu patients with haemosiderosis and in rats subjected to iron overload (Powell *et al.*, 1975b). However, in a patient with haemochromatosis, who had been treated by phlebotomy and who was iron-deficient

at the time of death, the isoferritin pattern had reverted to normal. In transfusional haemosiderosis and alcoholic cirrhosis the isoferritin pattern showed a normal organ distribution (Powell *et al.*, 1974).

Although the varied isoferritin and, possibly, subunit composition may explain the difference between various tissue ferritins, there remains the intriguing question as to whether this has any physiological significance. In the present study human ferritin fractions have been isolated and investigated with regard to their biochemical and immunological properties.

Methods and Materials

Tissues

Normal tissues were obtained at post mortem from healthy subjects dying after road accidents. Tissues were also obtained from patients with aplastic anaemia or thalassaemia who died after transfusional iron overload.

Preparation of ferritin

Ferritin was prepared by the method of Worwood *et al.* (1975). As this procedure selects iron-rich ferritin, some samples were prepared without ultracentrifugation, but with the addition of a second gel-filtration step on Sepharose 6B [Pharmacia (G.B.), London W.5, U.K.]. Purity was confirmed by polyacrylamide-gel electrophoresis and analytical isoelectric focusing. Liver ferritin was also prepared by methods in which one stage of the whole preparative procedure was omitted. These were (a) heat treatment at 70°C, (b) acid precipitation, (c) precipitation with $(\text{NH}_4)_2\text{SO}_4$, and (d) ultracentrifugation. Each preparation showed microheterogeneity on isoelectric focusing and any differences seen were quantitative rather than qualitative.

Isoelectric focusing

Analytical isoelectric focusing on polyacrylamide-gel cylinders was carried out as described previously (Worwood *et al.*, 1976a). Analytical isoelectric focusing in 5% polyacrylamide slabs was performed by the method of Drysdale (1974) by using the MRA slab-gel apparatus (MRA Corp., Boston, MA, U.S.A.) with 3.6% Ampholine (36% pH 4–6 range, 54% pH 5–7 range and 10% pH 3.5–10 range; LKB Instruments, South Croydon, Surrey, U.K.). Samples containing 10–20 µg of ferritin protein were loaded into slots in the gel in a volume of 5 µl. More dilute samples were applied in succession at 10 min intervals. A current (maximum 300 V, 2 W) was passed for 6 h. The gels were fixed for ½ h in 5% (w/v) trichloroacetic acid and washed overnight in water to remove the ampholytes. The gels were stained for protein

(10 µg samples) or iron (20 µg samples) (Worwood *et al.*, 1976a).

Preparative disc-gel isoelectric focusing was performed in the MRA disc-gel apparatus fitted with 140 mm × 12 mm preparative gel tubes. Liver ferritin was focused in 4% acrylamide with 2% pH 5–7 ampholyte, and heart ferritin in 2% pH 4–6 ampholyte. Focusing took about 12 h at 800 V, 5 W maximum, but was continued for a further 5–6 h to ensure that equilibrium was attained. The ferritin bands were cut out, chopped into fragments and eluted in 1–2 vol. of boiled water, the pH being measured at the running temperature of the gel (4–6°C). The gels were further eluted with 2 × 20 ml of 0.05 M-phosphate buffer, pH 7.4, for 48 h. All three eluates were pooled, concentrated by ultrafiltration and checked by analytical isoelectric focusing. Each fraction was refocused and, if necessary, focused a third time. Finally, the ampholytes were removed by gel filtration on Sepharose 6B. In all cases focusing was carried out in a cold-room at 4–6°C and the gels were further cooled by circulating water at the same temperature.

Anion-exchange chromatography

Anion-exchange chromatography was carried out as previously described (Worwood *et al.*, 1975) on Sephadex A-50 at pH 6.8.

Protein determination

Protein was measured by the method of Lowry *et al.* (1951), with bovine serum albumin as a standard, or sometimes, for apoferritin, by measuring A_{280} ($A_{1\text{cm}}^{1\%} = 9.0$) (Macara *et al.*, 1972).

Iron content of ferritin

This was estimated by flameless atomic-absorption spectrometry (Varian model 1100 and model 63; Varian Techtron Pty., Melbourne, Vic., Australia) or by its absorption at 420 nm ($A_{1\text{cm}}^{1\%} = 67$, uncorrected for light-scattering). The latter value was calculated by comparing the iron content estimated by atomic-absorption spectrometry with the absorption at 420 nm on human heart, kidney and liver ferritins. It is somewhat lower than that calculated for horse spleen ferritin (Macara *et al.*, 1972).

Ferritin assay and immunoreactivity

Ferritin was assayed by the immunoradiometric method of Jones & Worwood (1975), by using antibody against spleen ferritin, or by the heart ferritin assay of Jones & Worwood (1978). Immunoreactivity is expressed as a ratio of ferritin concentration determined by the immunoradiometric assay and that by the method of Lowry *et al.* (1951).

Preparation of apoferritin

Apoferritin was prepared by the reduction of ferritin with sodium dithionite (Macara *et al.*, 1972). As ferritin tended to be precipitated in high concentration it was diluted to 1–2 mg/ml.

Iron uptake

The method of Macara *et al.* (1972) is not suitable for use with ferritin concentrations of less than 250 µg/ml. Uptake is very slow with the Bryce & Crichton (1973) method at the concentrations used here, so the method described below was developed.

Experiments were carried out in barbitone sodium/HCl buffer at 20–22°C with ferrous ammonium sulphate and molecular oxygen as oxidant. To establish the procedure preliminary experiments were performed on liver apoferritin by varying the following conditions.

(a) *pH*. This was varied from pH 6.0 to 7.5 at 1.0. Below pH 6.8 the system was unbuffered. The apoferritin concentration was 100 µg/ml and the iron concentration was 1000 atoms/ferritin molecule.

(b) *Ionic strength*. This ranged from 0.01 to 0.08. The contribution by Fe^{2+} was not calculated, but was constant in all experiments. The apoferritin concentration was 50 µg/ml and iron (500 atoms/ferritin molecule) was added in the appropriate buffer. Experiments were carried out at pH 7.0.

(c) *Apoferritin concentrations*. These covered the range 25–200 µg/ml in the presence of 2000 Fe atoms/ferritin molecule at pH 7.0, in 1.0. buffer.

(d) *Iron/protein ratio*. Between 1000 and 3000 atoms of Fe were added to apoferritin (50 µg/ml) at pH 7.0, in 1.0. buffer.

In the final method 52.5 µg of apoferritin (which

had been dialysed against the buffer) was added to barbitone sodium/HCl buffer, pH 7.0, 1.0. The buffer was prepared by mixing appropriate volumes of barbitone sodium (1.0) and HCl (1.0). Ferrous ammonium sulphate in water was added in not more than 50 µl so as not to decrease the buffer capacity to any extent. The final volume was 1.2 ml. Iron uptake was monitored at 420 nm in an SP.1800 spectrophotometer (Pye–Unicam, Cambridge, U.K.). As turbidity was a problem when high iron/protein ratios were attained the samples were placed in a position close to the photoelectric cell to minimize light-scattering effects. In the kinetic experiments a single batch of Fe^{2+} was added and the reaction followed to completion. For total uptake the Fe^{2+} was added in stages, the amount of iron in the batches being decreased as the end point was approached. For example, in one experiment the equivalent of 1000 atoms of Fe/ferritin molecule was added and followed in succession by 500 Fe atoms/molecule and two batches of 200 Fe atoms/molecule.

In some experiments uptake of $^{59}\text{Fe}^{2+}$ was monitored. [^{59}Fe]Ferrous ammonium sulphate was produced by equilibrating $^{59}\text{FeCl}_3$ with a 5000-fold excess of non-radioactive Fe^{2+} (Hoy *et al.*, 1974). After the reaction was complete desferrioxamine was added at twice the molarity of the original Fe^{2+} concentration, and the mixture exhaustively dialysed against barbitone sodium/HCl buffer, pH 7.0, 1.0.

Results

Separation of isoferritins

Normal liver ferritin (Fig. 1) and ferritin from a patient with transfusional iron overload were separated into six isoferritin fractions, some of which

Fig. 1. Slab analytical isoelectric focusing of liver ferritin fractions previously separated by preparative disc isoelectric focusing

(a) Whole ferritin; (b)–(g) isoferritin fractions. The mean pI values for fractions (b)–(g) are given in Table 1, fraction (b) having a mean pI of 5.34. The bands are stained for protein. See the text for experimental details.

contained more than one band. The analytical isoelectric focusing pattern of both preparations was identical. It proved difficult to obtain single bands even after three preparative isoelectric focusing runs, which decreased the overall recovery of ferritin to less than 5%, but it is worth noting that ten or more bands may be distinguished in a range of 0.5 pH unit (Fig. 2). Isoelectric points are given in Table 1. Measurements at room temperature gave values approx. 0.1 pH unit lower and may account for the differences between pI values found here and those published elsewhere (Drysdale, 1974; Powell *et al.*, 1975a).

Preparative isoelectric focusing was used initially to separate two or three bands of heart ferritin focusing at the mean pI of 5.05. Later, ion-exchange chromatography was used, as it gave almost as good discrimination as isoelectric focusing, but with a better recovery.

Fig. 2. Slab analytical isoelectric focusing of human liver ferritin stained for protein

At least ten isoferritin bands can be distinguished. For experimental details see the text.

Immunoreactivity

The liver isoferritins reacted decreasingly with the antibody against heart ferritin from the acidic to the more alkaline end of the spectrum (Table 1). There was a converse situation with the antibody against spleen ferritin, except that there was a fall in reactivity for the most alkaline fraction. This was observed in two separate batches of isoferritins and was confirmed by the Laurell (1972) 'rocket' technique.

Iron content of isoferritins

With protein derived from liver the iron/protein ratio was highest in the isoferritin with the lowest pI and gradually fell with increasing pI (Table 1).

Ion-exchange properties of isoferritins

The ion-exchange affinity of the isoferritin fractions followed the same pattern as the pI values. The middle fractions were eluted from the ion-exchange column at a chloride ion concentration of about 0.20M, which agreed with the peak of elution for whole liver ferritin.

Iron uptake by whole liver apoferritin

Uptake of iron was low at pH6.0 and increased with rising pH (Fig. 3a). Above pH7.0 there was a marked increase in the tendency to precipitate and by pH7.5 precipitation of ferritin occurred almost instantaneously. This was confirmed by a decrease in the protein content of the supernatant. At pH6.7–6.8 the rate of uptake was moderate and the tendency to precipitate was low, but this was at the extreme of the buffer range. A compromise pH of 7.0 was

Table 1. *Properties of liver and heart ferritin fractions*

Heart ferritins underwent an ultracentrifugation step that selected iron-rich molecules. The iron/protein ratio does not therefore relate to the original iron content and is not included in the Table. Numbers of determinations are given in parentheses. See the Methods and Materials section for experimental details.

Ferritin	pI (mean $\pm$ S.D.)	Immunoreactivity [protein concn. (immunoradiometric assay)/protein concn. determined by the method of Lowry <i>et al.</i> (1951)]		Fe/protein ratio† (μ g of Fe/ μ g of protein)	Anion-exchange affinity of separated fractions† [concn. of Cl ⁻ (M) at the peak ferritin concn.]
		Spleen assay	Heart assay		
Heart	5.05† (3)	0.036 (2)	1.0 (2)	—	0.290 (2)
Liver	5.34* $\pm$ 0.02 (12)	0.38 (3)	0.030 (2)	0.24 (2)	0.248 (2)
	5.43* $\pm$ 0.04 (18)	0.74 (3)	0.025 (2)	0.22 (2)	0.240 (2)
	5.57* $\pm$ 0.03 (14)	1.03 (3)	0.015 (2)	0.18 (2)	0.214 (2)
	5.67* $\pm$ 0.03 (10)	1.25 (3)	0.010 (2)	0.14 (2)	0.190 (2)
	5.74* $\pm$ 0.01 (5)	1.35 (3)	0.005 (2)	0.10 (2)	0.155 (2)
	5.81* $\pm$ 0.01 (8)	0.79 (3)	0.003 (2)	0.07 (2)	0.108 (1)

* Mean values for ferritin from normal tissues and those of patients with transfusional iron overload. There were negligible differences between normal and iron-loaded tissues.

† Values for ferritin from normal tissues only.

used in the final method. Increasing concentrations of apoferritin increased the rate (Fig. 3b). The buffer capacity, however, was not high and the limit of apoferritin concentration at pH 7.0 was about 100 $\mu\text{g/ml}$ with 2000 atoms of iron/molecule. At a concentration of 200 $\mu\text{g/ml}$ and the same proportion of iron, the pH fell and the rate of iron uptake fell accordingly. The response to varying the ionic strength (Fig. 3d) followed Brönsted-Bjerrum kinetics, which hold for ionic protein binding (Macara *et al.*, 1972; Wagstaff *et al.*, 1977) as well as salt interactions. As expected, the rate increased as the proportion of iron was raised (Fig. 3c).

In all these experiments the rate curves were sigmoid when iron was added to apoferritin, as found by Macara *et al.* (1972), but subsequent additions of iron resulted in hyperbolic rate curves. After an initial lag period uptake by apoferritin was rapid, and on addition of further iron the initial rate rose, but after several additions it began to fall. At around 1500 atoms/molecule the solution became turbid, the turbidity increasing as more iron was added, until finally there was overt precipitation of ferritin at about 2500–3000 atoms/molecule.

On addition of relatively small amounts of iron to liver apoferritin the reaction did not appear to go to

Fig. 3. Effect of varying conditions on the uptake of iron by liver apoferritin

(a) pH; (b) protein concentration; (c) ferrous iron concentration; (d) ionic strength. The numbers by each experimental curve indicate pH in (a), ferritin protein ($\mu\text{g/ml}$) in (b), number of iron atoms added to each ferritin molecule in (c) and ionic strength in (d).

Table 2. Iron uptake by unfractionated liver apoferritins

Iron uptake at low concentrations of apoferritin (52.5 μg in 1.2 ml). Uptake is incomplete at low iron concentrations, but as the concentration of iron is increased a greater proportion of it is taken up by the apoferritin.

Total number of iron atoms added/apoferritin molecule	Uptake (Fe atoms/ferritin molecule) determined from A_{420}	Uptake (%), determined by A_{420}	Uptake (Fe atoms/ferritin molecule) determined by ^{59}Fe uptake	Uptake (%), determined by ^{59}Fe
500	348	70	378	76
1000	758	76	842	84
1500	1309	87	1359	91
2000	1880	94	1872	94

completion when monitored at 420 nm. As more iron was added a greater proportion of iron was taken up by the ferritin. Reaction with 2,4,6-tripyridyl-sym-triazine showed that no ferrous iron was left in solution. The experiments were repeated with [^{59}Fe]ferrous ammonium sulphate as iron donor. The results showed the same trend (Table 2). Iron was not removable by dialysis against 0.05 M-barbitone sodium/HCl buffer, pH 7.0, but desferrioxamine, an iron chelator, readily removed some of the iron. This suggests that at low iron and apoferritin concentrations, iron is adsorbed to the outside of the ferritin molecule.

Liver ferritin samples from these experiments were subjected to isoelectric focusing and stained for iron. At low concentrations the iron was preferentially taken up by the most acidic isoferritins and, as the proportion of iron was increased, so did the more alkaline isoferritins take up the iron. As more iron was added, the iron content of the most acidic isoferritins appeared to fall. When the gels were stained for protein this was shown to be due to loss of protein, presumably by precipitation, rather than redistribution of the iron. This probably accounts

for the turbidity which appears at about 1500 iron atoms/ferritin molecule.

Similar experiments were carried out with heart ferritin obtained at post mortem from a patient with thalassaemia and iron overload. The ferritin contained, in addition to the typical bands of acidic isoferritins from heart (Figs. 6*n-6p*), a whole range of isoferritins corresponding to those in liver. With concentrations of iron up to 2250 atoms/ferritin molecule, most of the iron went into the acidic bands.

Iron uptake by apoferritin prepared from ferritin fractions

The total uptake of iron was determined with the isoferritin preparations obtained by isoelectric focusing and converted into apoferritin by sodium dithionite reduction. As the amount of protein available was limited, kinetic studies were performed with fractions separated by anion-exchange chromatography. The apoferritins prepared after isoelectric focusing took up between 1800 and 3000 atoms of iron/ferritin molecule before precipitating. A preparation of acidic heart apoferritin took up 3500 atoms of iron/molecule. It is noteworthy that

Fig. 4. Uptake of iron by various apoferritins and apoferritins prepared from ferritin fractions obtained by ion-exchange chromatography

Iron (1000 atoms/ferritin molecule) was added as ferrous ammonium sulphate to apoferritin (52.5 μg) and the reaction followed by the A_{420} . The inset diagram shows the range of isoferritins present: (a)–(c) normal heart ferritin; (d) heart ferritin in transfusional iron overload (thalassaemia); (f) unfractionated liver ferritin; (e), (g) and (h) liver ferritin separated by ion-exchange chromatography and eluted at 0.255 M-, 0.205 M- and 0.170 M- Cl^- respectively.

Fig. 5. Time taken for 50% iron uptake by apoferritin ($t_{1/2}$) in relation to the peak $[Cl^-]$ on ion-exchange chromatography

Δ , Heart ferritins; $\circ$, liver ferritin fractions separated by ion-exchange chromatography; $\bullet$, unfractionated liver ferritin. The correlation coefficient (r) was 0.89.

apoferritin prepared from the most alkaline liver ferritin, which corresponds in pI to the 'natural apoferritin' of Arioso *et al.* (1977), was able to take up 2600 atoms of iron/molecule before precipitation.

The kinetic experiments were carried out with four preparations, three from normal heart ferritin subjected to ultracentrifugation to select the more (iron-rich) acidic bands, and one from the heart of a patient with iron overload, which was not ultracentrifuged and had a broad spectrum of iso-ferritins. Liver ferritin was subjected to ion-exchange chromatography and the rate of uptake measured on seven fractions after reduction to apoferritin. Fig. 4 shows that the slowest rate was in the liver fraction eluted at low chloride concentrations (and hence high pI), whereas the fastest rate was in the fractions eluted at high chloride concentrations (low pI). Higher rates were found with the heart apoferritins, and the slowest of these was in the apoferritin prepared from the patient with iron overload. Fig. 5 shows that there is good correlation between the rate of uptake and pI of the iso-ferritins.

Uptake by the more basic apoferritin fraction appeared to follow sigmoid kinetics, suggesting less

Fig. 6. Slab isoelectric focusing profiles of ferritin prepared from normal and iron-overloaded human tissues. Each profile of a particular tissue ferritin was obtained from a different subject. The gels were stained for iron; (a) and (e), normal kidney; (b)–(d) kidney (transfusional iron overload); (f) normal liver; (g)–(j), liver (transfusional iron overload); (k)–(m) heart (transfusional iron overload); (n)–(p) normal heart; (q) and (s) normal spleen; (r) spleen (transfusional iron overload). The profiles of ferritins prepared from spleen, liver and kidney of patients with transfusional iron overload showed little difference from those of ferritin isolated from the corresponding normal tissues. However, ferritin prepared from the hearts of patients with iron overload showed an increase in the more basic iso-ferritins compared with ferritin prepared from normal heart. It can be seen that the isoelectric focusing profiles of normal heart are very variable.

efficient nucleation (Macara *et al.*, 1972) than in the acidic apoferritins (Fig. 4). The slope of the latter curves were considerably steeper than the former.

Isoferritin patterns and iron content of iron-overloaded and normal tissues

Ferritins were prepared from tissues of patients with iron overload resulting from multiple blood transfusions. The recovery of ferritin from such tissues was 2–20-fold that of normal heart tissues. These preparations were subjected to isoelectric focusing and compared with normal ferritin (Fig. 6). Heart ferritin showed a relative increase in the concentration of basic isoferritins. There was no decrease in the amount of acidic isoferritins, and when the large increase in total ferritin was taken into account there was a considerable increase in absolute terms. Isoelectric focusing profiles of iron-loaded liver and kidney and spleen ferritins showed little difference from that of the normal ferritin.

Iron/protein ratios were measured after ion-exchange chromatography on ferritin extracted from an iron-overloaded and a normal heart. There was an increase in iron content in ferritin from both tissues as the salt content of the elution buffer increased. The lowest iron/protein ratios (μg of Fe/ μg of protein) were in the most basic fraction in both cases (0.06:1 in normal heart and 0.085:1 in iron-overloaded heart), eluted at 0.13M-Cl^- , and the maximum iron contents were 0.145:1 at a Cl^- concentration of 0.24M in the normal heart and 0.49:1 at 0.25M-Cl^- in the iron-overloaded heart ferritins. The peak protein concentrations of the acidic isoferritins were eluted at 0.28M- and 0.305M-Cl^- respectively. Isoelectric focusing showed a preponderance of the more basic of the two major acidic isoferritins in both samples. Later fractions, in which the most acidic heart ferritins predominated, had slightly lower iron/protein ratios.

Discussion

The reactivity of isoferritins against antibodies to spleen and heart ferritin is related to pI and affinity on ion exchange. The one exception to this is the most alkaline ferritin. The acidic isoferritins react more strongly than the more basic isoferritins with an antibody raised to heart ferritin. The opposite effect is seen with an antibody to spleen ferritin and in both cases there is a gradient of immunoreactivity with pI. The most obvious explanation of these findings is that the two antibodies are directed against two distinct sites, heart-antibody-reacting sites being more abundant in the acidic isoferritins and spleen-antibody-reacting sites in the more alkaline isoferritins. This fits in well with the two-subunit hypothesis, each antibody being directed against a subunit type. The iron content of iso-

ferritins also varies with pI. This is not necessarily related to a structural difference in the isoferritins, but the uptake experiments on apoferritin prepared from liver and heart and on partially separated isoferritin fractions show that the most acidic isoferritins gain iron more readily than the alkaline ones.

The increase in rate constant and, possibly, more efficient nucleation in the more acidic ferritin can partially explain the iron distribution in the isoferritins, the more acidic species taking up iron at a faster rate than the more basic. In liver ferritin, where the proteins at the extreme end of the spectrum are in low concentration, the most basic isoferritin will always contain very little iron, because the rate constant is comparatively low and the protein concentration relative to the major isoferritins is also low. At the acidic end of the spectrum the rate constant is high, but the iron content of the most acidic isoferritin will depend on the relative protein concentration.

Harrison *et al.* (1977) showed that horse spleen apoferritin prepared from ferritin of high initial iron content takes up iron at a faster rate than that prepared from ferritin of low iron content, an analogous situation to that described here. As natural apoferritin from horse spleen is the most acidic isoferritin (Ishitani *et al.*, 1975) it would be interesting to see if horse heart ferritin, which has a higher pI than horse spleen ferritin (Drysdale, 1977) takes up iron at a faster rate.

The isoelectric focusing patterns of ferritin isolated from iron-loaded heart are more in keeping with the results of Bomford *et al.* (1977) for ferritin from patients with haemochromatosis than with those of Powell *et al.* (1974). Far from there being a decrease in the concentration of acidic isoferritins, there was a considerable increase in the total content, but with a greater relative increase in the more basic ones. In addition there was a marked increase in the iron content of the ferritin, especially towards the acidic end of the spectrum, where it approached saturation (4000 Fe atoms/ferritin molecule). Our experience agrees with that of Bomford *et al.* (1977) that normal heart ferritins are highly variable in their isoferritin profiles. The only constant feature appears to be the presence of isoferritins around pI 5. Occasionally a preparation will contain these bands alone, but generally the more acidic of the liver-type ferritins are present, and sometimes the whole range. Nevertheless, in the iron-overloaded heart ferritins that we examined there was a definite increase in the extent and amount of more basic isoferritins.

It has been suggested that in rat liver iron induces the synthesis of L subunits (Adelman *et al.*, 1977) and it is proposed that preferential stimulation of L-subunit synthesis is a general mechanism leading to the disappearance of the more acidic isoferritins

in iron-overloaded tissue (Drysdale, 1977). Our studies of heart muscle do not support this hypothesis and suggest that in a tissue with a high proportion of acidic isoferritins iron initially induces H rather than L subunits. It is possible that with increasing concentration of iron there is a limit to H-subunit synthesis, followed by an increase in the proportion of L subunits. This would explain the reported variability of the isoferritin composition of heart muscle.

The present observations showing a compatibility between the iron content of isoferritins and iron uptake by the corresponding apoferritins, together with the isoferritin profiles of normal human tissues and those from patients with iron overload, are consistent with the hypothesis that these proteins are composed of variable proportions of different subunits. A direct demonstration of the relationship between the functional activity of ferritin molecules and their subunit composition would be desirable.

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