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Lysosomal and cytosolic ferritins

A biochemical and electron-spectroscopic study

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Summary. Cytosolic and lysosomal ferritin and haemosiderin were isolated from rat livers which had been iron-loaded by four intraperitoneal injections of iron-dextran. The cytosolic and lysosomal ferritins, prepared in a phosphate-free medium, were subjected to gel-filtration chromatography on Sepharose 6B, yielding four fractions: a cytosolic monomeric (CMF) and void-volume ferritin fraction (CVVF), and a lysosomal monomeric (LMF) and void-volume ferritin fraction (LVVF). Of each fraction the following aspects were examined: (a) immunoreactivity against specific antiserum; (b) the Fe/P mass ratio and the effect of dialysis on this ratio using electron probe micro-analysis (EPMA); (c) morphology and Fe-specific imaging using electron spectroscopic imaging (ESI) and electron energy loss spectroscopy (EELS). For haemosiderin one aspect, the Fe/P ratio, was determined before and after extensive purification. The following results were obtained (a) All ferritin fractions reacted with anti- (rat liver ferritin). (b) The Fe/P ratios as determined in CMF in an haemosiderin were not affected by dialysis or extensive purification, respectively. The Fe/P ratio in CVVF was affected by dialysis. In the lysosomal fractions, only a trace of phosphorus (LVVF) or no phosphorus (LMF) was detected. (c) Morphologically, CMF and CVVF were found to be rather homogeneous; the iron core diameters of both fractions were in the known size range. LMF and LVVF were of rather heterogeneous composition; the core diameters of these fractions were different. In conclusion: the phosphorus in ferritin and haemosiderin is firmly bound; Haemosiderin, when derived from ferritin, has to take up phosphorus in the lysosomes.

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Introduction

Ferritin and haemosiderin are two important iron-storage compounds present, amongst others, in animals (Wagstaff et al. 1982; O'Connell et al. 1987; Sindram et al. 1986). Ferritin consists of an iron-containing core, 6–7 nm in diameter, in which iron is present as a polynuclear ferric oxy-hydroxide–phosphate complex (Harrison et al. 1973; Harrison et al. 1974), and a surrounding protein shell. This shell, a complex of 24 polypeptide subunits, keeps iron in a soluble and non-toxic form. Normally, ferritin is mainly present in the cytoplasm of the cell, but in case of iron overload it is also found in the lysosomes (Jacobs 1985; Harrison et al. 1987; Mostert et al. 1989).

Haemosiderin is a purely lysosomal product which ultrastructurally resembles ferritin. The main difference between ferritin and haemosiderin is that the latter is water-insoluble, probably due to the lack of an intact protein shell. It consists of iron-containing granules visible in the electron microscope as irregular massive aggregates of electron-dense particles resembling ferritin iron cores. The particles are of irregular shape ranging over 1–7.5 nm in diameter (Wixom et al. 1980; Dickson et al. 1988).

Because ferritin and haemosiderin have similar X-ray diffraction patterns (Fischbach et al. 1971), similar Mössbauer spectra (Andrews et al. 1988) and similar appearances in the electron microscope (Wixom et al. 1980), it is generally assumed that haemosiderin is a degradation prod-

uct of ferritin. One theory (Hoy et al. 1981) maintains that haemosiderin is formed out of ferritin through a polymerization process. Such polymers may be represented by the cytosolic clusters of ferritin particles described amongst others by Myagkaya and de Bruyn (1982). Polymerized ferritin molecules may be incorporated in lysosomes and converted into haemosiderin. Changes at the surface of the ferritin molecules could be a signal for polymerization, and this a signal for incorporation into lysosomes. So, the most important step in the formation of haemosiderin from ferritin should be polymerization of ferritin molecules. Another theory (Richter 1983) suggests that decomposition of the protein shell of ferritin by lysosomal enzymes is a very important step in the formation of haemosiderin from ferritin. The divested iron cores of these 'siderosomal ferritins' are unstable and could easily be transformed into finer particles, typical of haemosiderin. However, a third view maintains that it is still not completely established that haemosiderin really is a product of ferritin (St. Pierre et al. 1988; Dickson et al. 1988).

From previous work (Andrews et al. 1988), we know that the iron/phosphorus ratios of siderosomal and cytosolic ferritins from iron-loaded rat livers, prepared in phosphate buffer, are influenced by dialysis. We now want to establish the iron/phosphorus mass ratios of the various ferritin fractions and haemosiderin, and the effect of dialysis (in the case of the ferritins) and further purification (in the case of the haemosiderin) on these ratios when phosphorus is excluded during the whole isolation procedure. In the work described here we have used a similar rat model, iron-loaded by intraperitoneal injections of iron-dextran, to investigate further the chemical and morphological differences between lysosomal and cytosolic ferritins and the way they resemble haemosiderin. The Fe/P ratios are determined by electron probe micro-analysis (EPMA). The morphological and element-specific analyses of the ferritin fractions are performed by electron spectroscopic techniques.

Materials and methods

Animals. Iron-loaded male Wistar rats weighing 300–320 g ($n = 16$) were obtained by giving four intraperitoneal injections of Imferon (Fisons, UK) in a total dose of 100 mg Fe(III) spread over a period of four weeks. After the last injection of Imferon, the rats received no further treatment for a period of four weeks (resting period). Immediately after the resting period the livers were processed.

Preparation of liver homogenate. Rats were anesthetized intraperitoneally with 0.7 ml pentobarbital (35 mg). The blood of the liver was washed out by a brief perfusion through the portal vein with 0.15 M NaCl, flow 20–25 ml/min. The liver was removed, weighed and cut in pieces; 0.15 M NaCl was added up to a concentration of 1 g tissue/10 ml 0.15 M NaCl. Homogenization of the liver was performed in a Potter-Elvehjem homogenizer (500 rpm, six complete strokes). One third of the homogenate was used for preparation of the cytosolic fraction and two thirds were used for the preparation of the lysosomal fraction.

Preparation of the cytosolic fraction. This fraction was prepared according to de Duve et al. (1955). The homogenate was centrifuged for 110 min at 70000 *g*, 4° C in a Beckman ultracentrifuge type L5-65, rotor 35. The supernatant, representing the cytosolic fraction, was frozen at −20° C.

Preparation of the lysosomal fraction. Lysosomes were prepared according to Hultcrantz et al. (1982) by the use of a continuous sucrose gradient (1.4–2.2 M) in a Beckman ultracentrifuge, rotor SW27. The pellet representing the lysosomal fraction, was resuspended in 0.3 M sucrose. Arylsulphatase (EC 3.1.6.1) activity in this fraction, as measured according to Milson et al. (1972), appeared to be seven times enhanced in comparison to the crude homogenate.

Preparation of haemosiderin. Haemosiderin was prepared in a way similar to, but not identical with, the method of Vidnes and Helgeland (1973). The lysosomal fraction was subjected to osmotic shock with bidistilled water, sonicated for 1 min with a vibra cell, position 5, and centrifuged for 10 min at 10000 *g*, 4° C in a Beckman centrifuge type J-21c, rotor Ja-20. The resulting supernatant was used for the preparation of lysosomal ferritin *(vide infra).* The pellet was resuspended in 6 vol. 0.15 M NaCl and centrifuged for 10 min at 10000 *g*, 4° C. This action was repeated once. The resulting pellet was resuspended in 2 vol. bidistilled water and sonicated for 1 min, position 5. This suspension was centrifuged for 10 min at 25000 *g* and the pellet was resuspended in bidistilled water. This was repeated twice. Next, the pellet was frozen in liquid nitrogen and thawed with bidistilled water to remove a gray-brown layer. The suspension was centrifuged for 10 min at 5000 *g*, 4° C. The pellet was resuspended in as little bidistilled water as possible and stirred for 24 h at 4° C. Finally, the suspension was centrifuged for 10 min at 3000 *g*, 4° C. The resulting pellet representing haemosiderin was resuspended in bidistilled water or in 0.15 M NaCl. Half of the haemosiderin was further purified by centrifugation through 4.1 M KI (McKay and Fineberg 1964).

Preparation of the ferritins. The cytosolic fraction and the supernatant of the sonicated lysosomes (see preparation of haemosiderin) were treated in the same way as for the isolation of ferritin. After heating for 10 min at 80° C and cooling on ice, both suspensions were centrifuged twice for 45 min at 3000 *g*, 4° C in a Beckman centrifuge type J-21c, rotor Ja-20. Supernatants were centrifuged in a Beckman ultracentrifuge type L5-65, rotor Ti-60 for 60 min at 78000 *g*, 4° C. Pellets were collected, suspended in 0.15 M NaCl and subjected to 7000 *g* for 60 min in the Ja-20 rotor. The resulting supernatants were centrifuged twice in the Ti-60 rotor for 60 min at 95000 *g*, 4° C and the pellets containing the ferritins were suspended in 0.15 M NaCl.

Chromatography. Gel filtration chromatography of the ferritins was performed on a Sepharose 6B column (4 cm × 91 cm)

in 0.05 M LiOH/borate pH 8.6. Fractions of 2.5 ml were collected and protein was monitored at 280 nm. Protein-containing fractions were pooled and half of the fractions was dialysed against bidistilled water for five days.

Protein determination. Protein was measured according to Markwell et al. (1987) at 660 nm.

Immunoreactivity. Each fraction was reacted with rabbit anti-(rat liver ferritin) specific antiserum (Mostert et al. 1989) in an Ouchterlony immunodiffusion assay.

Electron microscopy. Ferritins were air-dried on carbon-coated Formvar^R-filmed copper grids and examined in a Zeiss EM 902 transmission electron microscope. This instrument is equipped with an integrated electron energy spectrometer allowing, with energy-selected (e.g. zero loss) monospecific electrons, high-resolution contrast imaging (electron spectroscopic imaging, ESI) and element specific imaging (electron energy loss spectroscopy, EELS) (Ottensmeyer et al. 1980). In addition to using the EELS imaging mode, it is possible to acquire EELS spectra, comprised of element-specific edges, over a 'continuum' representing electrons which have suffered aspecific energy losses. In our case, iron was identified in the EELS spectrum by two Fe-specific edges at $\Delta E = 710$–730 eV. In the imaging mode it is possible to make semi-net intensity iron distribution images, by substracting the aspecific image (preabsorption image) from the element-specific image (made at the absorption edge). For further details see Hezel and Bauer (1987) and Cleton et al. (1989). Our image substractions and calculations of core diameters were accomplished by computer-assisted image processing with the aid of an IBAS/KONTRON 2000 (Mostert et al. 1989; Cleton et al. 1989). Approximately 100 iron core sizes were measured for particle size determinations.

Determination of Fe/P ratios by EPMA. Aliquots (5 µl) of each of the samples, comprising ferritins and sonicated haemosiderins, were dried on carbon-coated Formvar^R-filmed copper grids and analysed in a Philips EM 400 analytical electron microscope, operating at 80 kV from a LaB_6 electron source and equipped with a Tracor Northern, type TN2000 X-ray microanalyser. Multiple point analyses were performed at random over the grid squares essentially as described in Cleton et al. (1986). Peaks in the recorded spectra were identified by the available peak identification program. Net-intensity values for iron and phosphorus were obtained by the introduction of 400-eV-wide regions around the identified peaks and calculation by the energy filter program of the TN2000 computer software. Iron/phosphorus mass ratios were calculated from net intensities by the peak-ratio program.

Results

Chromatography

After separation on Sepharose 6B, cytosolic ferritin yielded a main peak at the position of monomeric ferritin. Hence we call this fraction cytosolic monomeric ferritin (CMF). A smaller peak eluted at the position of the void volume (called by us cytosolic void volume ferritin, CVVF). The position of the void volume was determined by blue dextran 2000. Lysosomal ferritin yielded a main peak at the position of the void volume fraction (called lysosomal void volume ferritin, LVVF) and a much smaller peak at the position of monomeric ferritin (called lysosomal monoferritin, LMF) (Fig. 1).

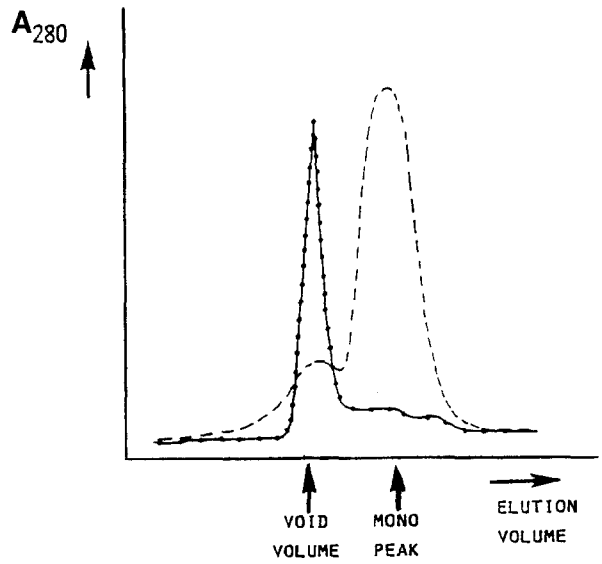

Fig. 1. Chromatography of lysosomal (-●-●-) and cytosolic (- - -) ferritin from iron-loaded rat livers on Sepharose 6B

Immunological identification

Protein concentrations of fractions which were used for immunological identification were as follows: 1.25 g/l (CMF), 0.75 g/l (CVVF), 0.50 g/l (LMF) and 2.00 g/l (LVVF). The cytosolic and lysosomal fractions both formed a single precipitin line in the Ouchterlony immunodiffusion assay against specific antiserum. Although the line formed by the LVVF was weak, it formed a single precipitin line that merged completely with the single line produced by the material from the CMF.

Table 1. Iron core diameters measured from ESI zero loss images

Fraction	Diameter (nm)		
	mean ±SD	mode	range
CMF	5.8±0.9	5.7	3.0
CVVF	5.8±0.9	5.7	3.0
LMF	5.6±1.0	4.3	3.8
LVVF	6.7±1.3	7.2	6.4

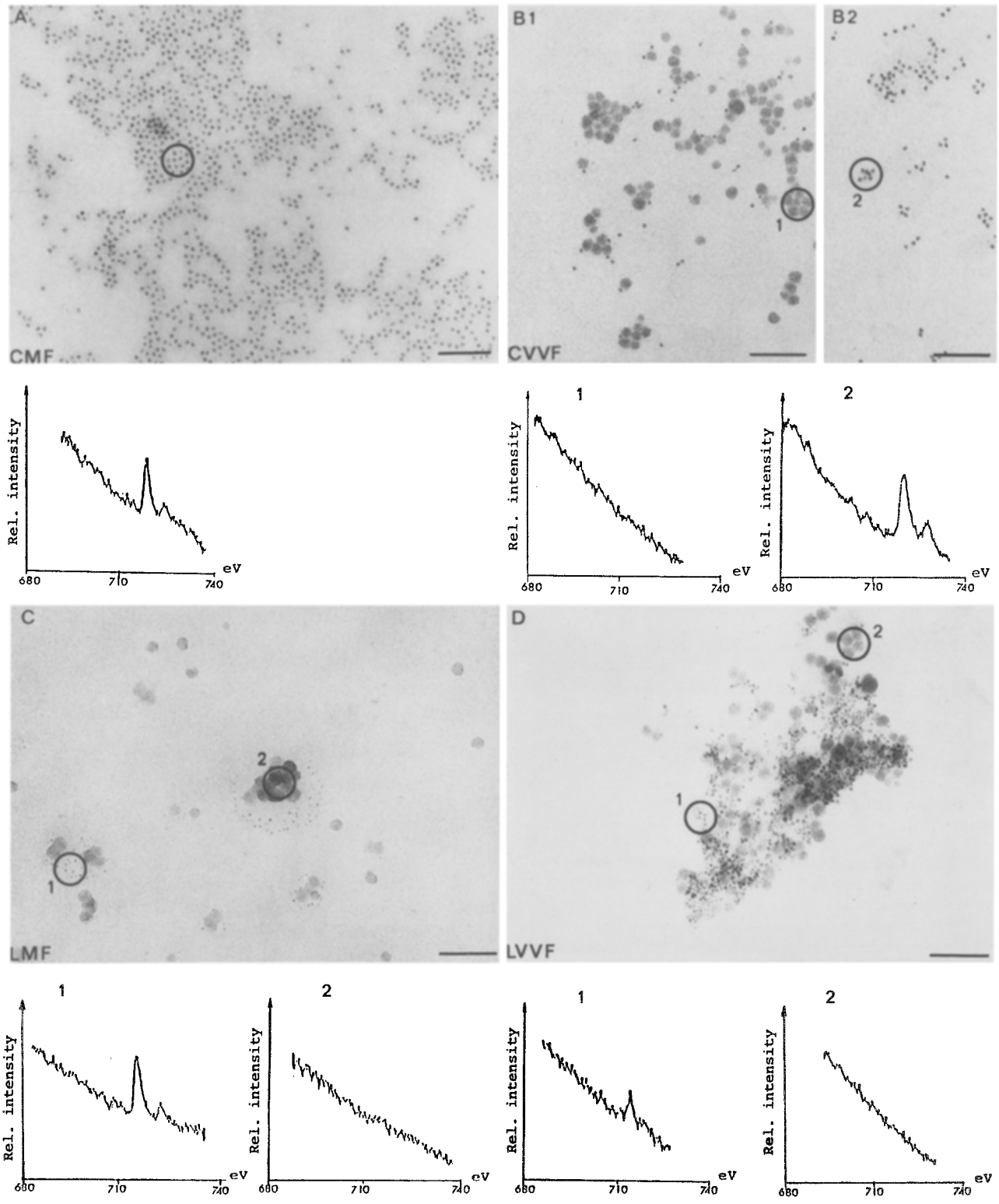

Fig. 2. ESI (zero loss) images ($\Delta E = 0$) of CMF **(A)**, CVVF **(B1+B2)**, LMF **(C)** and LVVF **(D)** with EELS spectra. The energy losses (horizontal axis) are plotted against the relative intensity (vertical axis). Bars represent 100 nm

Table 2. Fe/P mass ratios of iron-loaded cytosolic and lysosomal rat liver ferritin measured by EPMA

Fraction	Dialysis	Fe/P			
		mean ± SD	range	*n*	*t*-test
Cytosolic monopeak ferritin	–	34.6 ± 10.5	22.1–56.7	20	n.s.
	+	33.0 ± 8.5	18.7–48.3	20	
void volume	–	10.9 ± 3.7	5.9–15.9	20	$P<0.05$
	+	18.4 ± 4.2	13.5–25.5	20	
Lysosomal monopeak ferritin	–	no *P*	–	20	–
	+	–	–	20	–
void volume	–	no *P*/trace	–	20	–
	+	no *P*/trace	–	20	–

To obtain iron-loaded rat liver, four doses of Imferon^R were given. n.s. = not significant

Morphology

The morphology of each fraction was examined by electron spectroscopy. ESI (zero loss) images of the four fractions are presented in Fig. 2A–D. These images showed that all fractions, except for the CMF, contain two kinds of electron-dense particles of which the smaller ones resemble ferritin. EELS spectra revealed that the smaller particles indeed contain iron (see spectra under ESI images) and the larger particles do not. EELS semi-net intensity iron images corresponded with ESI zero loss images with respect to the localization of the ferritin-like particles, but not with respect to the size of the particles (CMF, see Fig. 3A, B). The mean size ± SD, mode and range of the ferritin cores as calculated from the ESI images by IBAS are summarized in Table 1.

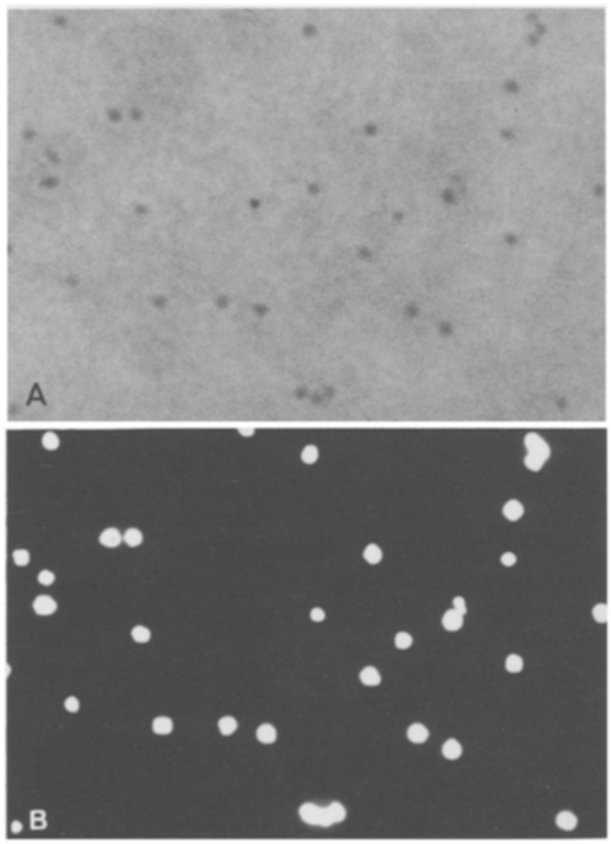

Fig. 3. ESI (zero loss) image **(A)** and EELS semi-net intensity iron image **(B)** of the CMF

Iron/phosphorus ratios

To study the presence of phosphorus and the effect of dialysis (ferritin samples) or further purification (haemosiderin), Fe/P ratios before and after treatment were determined by EPMA. The EPMA results are summarized in Tables 2 and 3. Fe/P ratios were established in CMF, CVVF and in haemosiderin; the values were found to be different in each of the three fractions. In LMF no phosphorus was detected and in LVVF only a

Table 3. Fe/P mass ratios iron-loaded rat liver measured by EPMA

Fraction	Fe/P			
	mean ± SD	range	*n*	*t*-test
Haemosiderin	16.1 ± 2.6	14.7–18.8	20	
Haemosiderin KI-purified	16.9 ± 3.1	12.1–18.9	20	n.s.

n.s. = not significant

trace. Dialysis had no significant effect on the Fe/P ratio of CMF but a slight effect on that of CVVF; extensive purification had no effect on the Fe/P ratio of haemosiderin.

Discussion

To determine atomic Fe/P ratios in cytosolic ferritin, lysosomal ferritin and haemosiderin, we preferred to use EPMA, as demonstrated before (Cleton et al. 1986), to the usual colorimetric techniques because the latter appeared to be not sufficiently sensitive to detect very low amounts of phosphorus. In our material, not prepared in phosphate buffer, there is a marked difference in the Fe/P ratios between cytosolic and lysosomal ferritins isolated from pooled iron-loaded rat livers. The lysosomal fractions contain only a trace of phosphorus (LVVF) or no phosphorus at all (LMF). In contrast, the cytosolic fractions CMF and CVVF and the haemosiderin do contain phosphorus. Assuming incorporation of cytosolic ferritin into the lysosomes, the results suggest that ferritin somehow, during this process, loses its phosphorus. When haemosiderin is formed, phosphorus is again attached to the ferric oxyhydroxide core. The mechanism, however, remains unclear. From in vitro studies it is known that iron cores are capable of adsorbing phosphate within lysosomes (Treffry and Harrison 1978; Treffry et al. 1987). Although the presence of acid phosphatase activity is acknowledged in iron-containing lysosomes (Sindram and Cleton 1986), a correlation between enzyme activity and the described phenomenon has not yet been demonstrated.

The Fe/P ratios found by us for the main ferritin fractions, i.e. the CMF (34:1) and the LVVF (no phosphorus), are similar to the ratios described by Andrews et al. (1988) for unfractionated cytosolic (36:1) and lysosomal ferritin (no phosphorus) prepared in phosphate buffer and dialyses for five days. Apparently, their material had taken up phosphate during the isolation procedure, which is in line with in vitro experiments of Treffry and Harrison (1978).

As the Fe/P ratios of the main cytosolic fraction CMF and the haemosiderin do not change significantly after extensive dialysis and further purification, respectively, we can conclude that the phosphorus in the ferritin and haemosiderin as prepared by our method is firmly bound. The change in the ratio in the CVVF, which is a fraction of all particles larger than 2000 kDa, may be due to phosphorus-containing compounds in which the phosphorus is affected by dialysis.

Previous work with ferritin in ultrathin sections showed a good correlation between core size measurements on ESI (zero loss) and EELS images (not published), and global (TEM) and EELS images (Cleton et al. 1989). In this study, with air-dried ferritin preparations, a difference was found between the measurements on ESI (zero loss) and EELS images. This observation is now subject to further investigation in our laboratory. As the ESI (zero loss) measurements on isolated cytosolic ferritin correlated well with our previous measurements on cytosolic ferritin in ultrathin sections, we used ESI (zero loss) images for core size determinations in this comparative study.

The zero-loss images reveal that both cytosolic fractions are rather homogeneous (range 3.0 nm); measurements of the iron core size of the particles (mean 5.8 ± 0.9 nm; mode 5.7 nm) are in line with data presented by Andrews (1986) and Andrews et al. (1988). The LVVF fraction was heterogeneous (range 6.4 nm) and most of the particles were larger (mean 6.7 ± 1.3 nm; mode 7.2 nm). The LMF fraction is rather homogeneous (range 3.8 nm), but the core sizes of the particles which occur most are smaller (mean 5.6 ± 1.0 nm; mode 4.3 nm). This fraction may represent the fast ferritin with an iron content which is lower than that of cytosolic ferritin as described by Andrews et al. (1987a, b). However, the complete LMF molecule cannot be smaller than the CMF molecule, because it elutes at a position similar to monomeric ferritin (Fig. 1). Obviously, when incorporation into the lysosomes occurs, parts of the iron-containing cores of the mono ferritin are lost. The larger particles as present in LVVF (see Table 1) might be aggregates of 'naked ferritin iron cores'. Our results support the theory of Richter (1984) that disintegration of the iron cores is part of the transformation of ferritin into haemosiderin.

It is possible that the decrease of the iron core diameter and the loss of phosphorus are events that are correlated. The effect of the phosphate may be to keep the iron in the ferritin cores together.

As the immuno-diffusion precipitin lines formed by CMF and LVVF merge completely, we can assume that these fractions are immunologically the same. Regarding the fact that the LVVF shows a large absorbance peak at 280 nm but forms only a weak precipitin line in the Ouchterlony assay, two explanations are possible: (a) another protein must be present in this fraction or

(b) the fraction consists of a protein that has lost almost all its ferritin epitopes and therefore cannot react with specific antiserum against rat liver ferritin. Both suggestions are in line with the observations that (a) in iron overload, an increased iron content in lysosomes is not accompanied by an increased immunoreactivity (Cooper et al. 1988) and (b) most of the particles in lysosomes that have the appearance of ferritin iron cores represent ferritin iron cores divested of protein or are surrounded by denatured protein (Richter 1984).

Very intriguing is (as established by EELS) the presence of the large, iron-deficient material in all fractions except the CMF. Preliminary studies show that the ferritin can be separated from the large particles by chromatography on a Sepharose 4B column.

In summary, our experiments have shown that, in the cytosol of iron-loaded rat livers, mainly monomeric ferritin is present. This monomeric ferritin reacts with specific antiserum, has a normal appearance in the electron microscope and contains phosphorus. Once isolated under phosphate-free conditions, the Fe/P ratio is not affected by dialysis. In the lysosomes of iron-loaded rat livers very little monomeric ferritin is present. This fraction, which reacts with specific antiserum, contains only a trace of phosphorus and its iron cores are smaller than normal ferritin iron cores. Neither of the lysosomal ferritin fractions contains phosphorus in contrast to haemosiderin, in which the amount of phosphorus has doubled compared to cytosolic ferritin. Hence, any haemosiderin that was derived from ferritin must have taken up phosphorus in the lysosomes. If not, another explanation could be that the haemosiderin and the lysosomal ferritin were unknowingly isolated from different liver cell types (hepatocytes and sinusoidal cells). Different cell types might acquire lysosomal phosphate from different sources (Weir et al. 1984) or might process iron-dextran in different ways or at different rates. To investigate this hypothesis a comparative study on ferritins and haemosiderin of isolated rat hepatocytes and sinusoidal lining cells is now being carried out in our laboratory.

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