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Analysis of iron-containing compounds in different compartments of the rat liver after iron loading

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Summary. The livers of iron-loaded rats were fractionated and a cytosolic fraction, a lysosomal fraction, a siderosomal fraction and haemosiderin were obtained. All iron-containing compounds from these fractions were isolated and their morphology, Fe/P ratios, iron core diameter and peptide content were compared. The cytosolic fraction contained ferritin (CF) and a slower sedimenting, light ferritin (CLF). The lysosomal fraction also contained ferritin (LF) and a slower sedimenting light ferritin (LLF). The siderosomal fraction contained ferritin (SF), a faster sedimenting non-ferritin iron compound (SIC) and haemosiderin (HS). SIC and HS did not resemble ferritin as much as the other products did, but were found to be water-insoluble aggregates. The Fe/P ratios of CF and CLF were lower than the Fe/P ratios of LF and LLF and these in turn had lower Fe/P ratios than SF, SIC and HS. The iron core diameter of the cytosolic ferritin was increased after lysosomal uptake. The iron core diameters of the siderosomal products were smaller. CLF, CF, LF, LLF and SF contained one kind of subunit of approximately 20.5 kDa. SIC and HS contained other peptides in addition to the 20.5-kDa subunit. The results indicate that storage of ferritin molecules is not limited to the cytosolic compartment, but is also the case in the lysosomes. Extensive degradation of the ferritin molecule seems to be confined to the siderosomes.

Key words: Electron spectroscopy – Ferritin – Haemosiderin – Iron-loaded rat – Morphometry

Introduction

One of the main characteristics of haemochromatosis is the presence of vast amounts of iron in liver, spleen and heart in the form of ferritin and haemosiderin (Richter 1978; Iancu et al. 1978; Sindram and Cleton 1986; Theil 1987). Both compounds contain an iron-oxyhydroxide-phosphate core in which a variable amount of iron is stored (Harrison et al. 1987). In ferritin the core is surrounded by a protein shell consisting of 24 polypeptide subunits that keeps the iron in a soluble form. In contrast to ferritin, haemosiderin is water-insoluble, probably due to the lack of parts of the protein shell (Weir et al. 1984). Another morphological difference is that haemosiderin cores are more irregular and smaller than ferritin cores (Wixom et al. 1980). It is generally assumed that haemosiderin is derived from ferritin as their iron cores have similar X-ray diffraction patterns (Fischbach et al. 1971) and their shells share peptides (Weir et al. 1984). The most likely site for the degeneration of ferritin into haemosiderin is the lysosomes, because lysosomes contain the enzymes for protein breakdown (De Duve and Wattiaux 1966) and it is the only place where ferritin and haemosiderin exist side by side. On the other hand, a lysosomal enzyme suitable for the digestion of ferritin has still not been found (Richter 1986; Hultcrantz et al. 1984). To investigate a possible transition process various studies of haemosiderin and ferritin have been performed in an iron-loaded rat model, such as Mössbauer spectra (Rimbert et al. 1985), iron release and uptake (Harrison et al. 1974; Brady et al. 1989; Biemond et al. 1984), the iron core composition and diameter (Treffry et al. 1987; Andrews et al. 1988a) and peptide content (Weir et al. 1984; Andrews et al. 1987). Yet it is still not entirely clear how and where a possible transition takes place (Hoy and Jacobs 1981; Richter 1986; Cooper et al. 1988).

Our previous chromatographic studies (Ringeling et al. 1989) in iron dextran-treated rats have shown that the diameter and the Fe/P mass ratios of the main cytosolic ferritin fraction were smaller compared to the diameter and the Fe/P ratios in the main siderosomal ferritin fraction. Moreover, Fe/P ratios of these ferritins could not be changed significantly by extensive dialysis. In this study, we fractionated the livers of similarly treated rats based on the fractionation scheme of Richter (1984). This allowed us to isolate, besides hae-

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mosiderin, a cytosolic fraction, a lysosomal fraction and a siderosomal fraction in one procedure. From these fractions we isolated all iron-containing products. By comparing their iron and protein characteristics, we followed the processing of cytosolic ferritin in lysosomes and siderosomes.

Materials and methods

Animals. Iron-loaded male Wistar rats weighing 300–320 g ($n = 12$) 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 the rats received no further treatment for a period of four weeks (resting period). Immediately after the resting period, the livers were processed.

Fractionation of the liver and isolation of iron-containing compounds. 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, frozen in liquid N_2 and stored at $-20°$ C until fractionation. For additional experiments fresh livers were used. Fractionation was performed based on the method of Richter (1984), see Fig. 1. Briefly, the homogenate, prepared in 0.3 M sucrose, was spun down twice yielding a supernatant, representing the (ferritin-containing) cytosolic fraction, and a pellet. This pellet was layered on a discontinuous sucrose gradient (1.4–2.2 M) yielding a pellet (the heavy lysosomes = siderosomes) and several bands, including the lighter lysosomes. Bands at least 1.5 times enriched in lysosomal arylsulphatase activity (EC 3.1.6.1; Milsom et al. 1972) were pooled. Both pellet and pooled bands yielded, after ultrasonic treatment and centrifugation, a pellet in which the haemosiderin was found and a supernatant, representing the (ferritin-containing) siderosomal and lysosomal fraction, respectively (Fig. 1). The haemosiderin from the siderosomes was further processed as described before (Ringeling et al. 1989), lyophilized and solubilized in 20 mM tetramethylammonium hydroxide (Weir et al. 1984). The haemosiderin yield from the lysosomes was too low to be analysed. Subsequently, the three ferritin containing fractions mentioned before (cytosolic, lysosomal and siderosomal) were processed using 0.15 M NaCl according to the procedure of Penders et al. (1968), see Fig. 2. In this procedure, we screened all intermediate and end products from this isolation scheme for the presence of iron-containing compounds. Water-soluble products were taken up in 0.15 M NaCl and water-insoluble compounds were solubilized in 20 mM tetramethylammonium hydroxide.

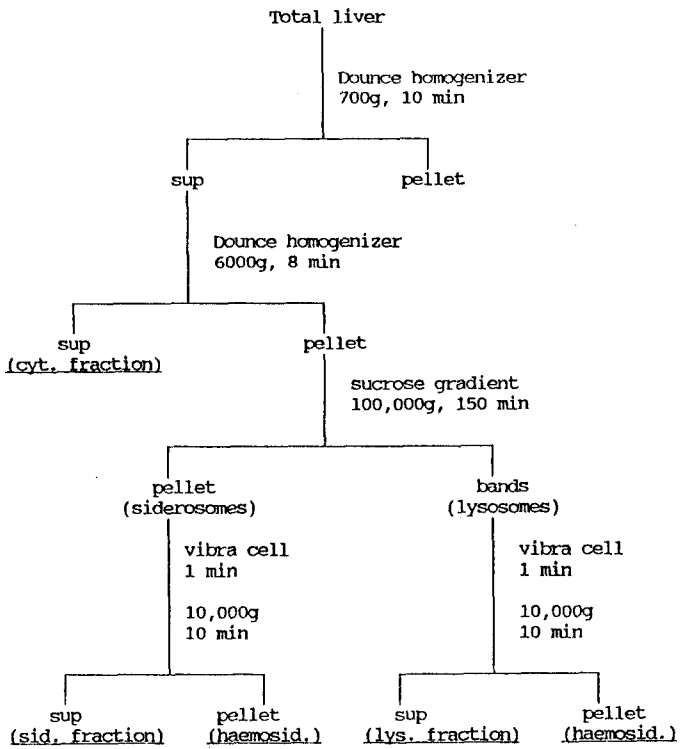

Fig. 1. Fractionation scheme for the preparation of the cytosolic, lysosomal and siderosomal fraction and the haemosiderin-containing fraction

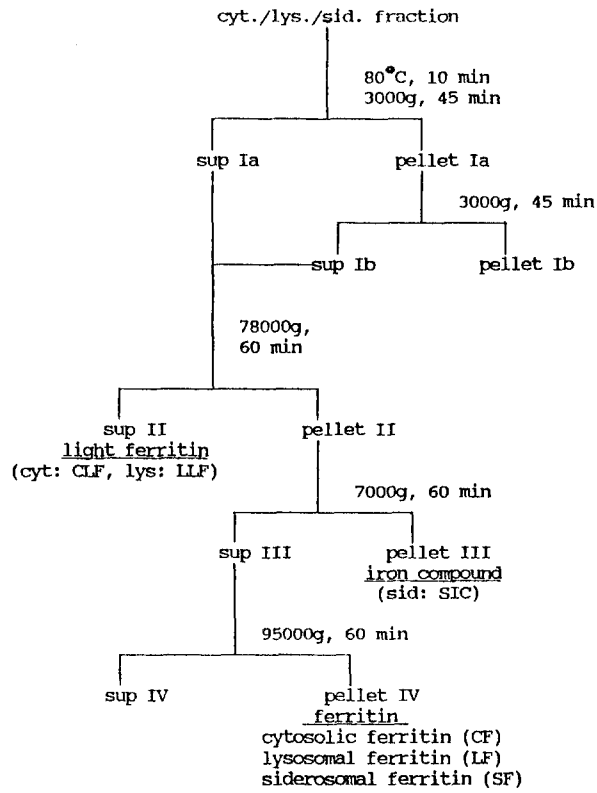

Fig. 2. Ferritin isolation scheme according to Penders et al. (1968)

Immunoreactivity. Each intermediate and end product of the above-mentioned procedure was reacted with rabbit anti-(rat liver ferritin) antiserum (Mostert et al. 1989) in an Ouchterlony immunodiffusion assay.

Determination of iron and protein. In all products iron concentration was determined according to the method of Harris (1978). Absorbance was measured at 562 nm. Ferritin protein was determined by radial immunodiffusion as described by Mancini et al. (1965) with rabbit anti-(rat liver ferritin) antiserum (Mostert et al. 1989).

Electron microscopy. All products 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-filtered (in our case zero loss) monospecific electrons, high-resolution contrast imaging (electron spectroscopic imaging, ESI) and element-specific imaging (electron energy loss spectroscopy, EELS) (Ottensmeyer and Andrew 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 peaks at $\Delta E = 710$–730 eV. For further details see Hezel and Bauer (1987) and Cleton et al. (1989). Calculations of core diameters were accomplished by computer-assisted ESI image processing with the aid of an Ibas/Kontron 2000 (Mostert et al. 1989; Cleton et al. 1989). Approximately 500–1000 iron cores per ferritin fraction were measured for particle size distribution. Diameters of particles of non-ferritin compounds were measured by hand from the ESI micrographs.

Determinations of Fe/P ratios by EPMA. Aliquots (5 µl) of each of the samples, comprising ferritins and solubilized non-ferritin compounds, were dried on carbon-coated Formvar^R-filmed 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 by 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.

Gel electrophoresis and Western blotting. Non-denaturing polyacrylamide gel electrophoresis (PAGE) was carried out in 5% gels using Tris/glycine pH 8.3 or in Pharmacia precast gradient gels 2/16 following manufacturer's instructions, in a Pharmacia GE 2/4 chamber. Gels were stained for protein with Coomassie brilliant blue G-250 and for iron with 2% $K_4Fe(CN)_6$ in 2% HCl. Ferritins, non-ferritin compounds and markers (Pharmacia HMW kit) were dissociated into subunits by heating (100° C, 5–10 min) in the presence of 2% (mass/vol.) SDS and 20 mM dithiothreitol just before electrophoresis. SDS/PAGE was carried out in 15% polyacrylamide gels using the Laemmli buffer system (Laemmli 1970) or in Pharmacia precast gradient gels 2/16. Gels were stained for protein with Coomassie brilliant blue G-250. For blotting, SDS/PAGE was carried out in 18% gels in a Bio-Rad Mini Protean II cell followed by electrophoretic transfer blotting in a Bio-Rad Mini Trans Blot cell. Proteins were stained with amido black, or blocked with gelatin and subsequently incubated with rabbit anti-(rat liver ferritin) antibody (Mostert et al. 1989) overnight. This was followed by washing and incubation with alkaline-phosphatase-conjugated goat anti-rabbit IgG (Bio-Rad). Immunostaining was performed according to the manufacturer's instructions.

Results

Iron compounds

From the cytosolic fraction two iron-containing products were isolated, one 'normal' ferritin end product (CF) and a intermediate product in the 78 000 *g* supernatant (Fig. 2, sup II). A similar result was obtained for the lysosomal fraction: a ferritin end product (LF) and an intermediate product in the 78 000 *g* supernatant (Fig. 2, sup II). The siderosomal fraction also yielded two products: the 'normal' ferritin end product (SF) and an intermediate product in the 7000 *g* pellet (Fig. 2, pellet III). In addition to the haemosiderin (HS) isolated earlier (Fig. 1), the end products and the intermediate products were tested in an Ouchterlony assay to determine their reaction with anti-(rat liver ferritin). Except for the haemosiderin and the intermediate product from the siderosomes, they all reacted with the antibody. Based on their positive reaction with specific antiserum we called the cytosolic intermediate product cytosolic light ferritin (CLF) and the lysosomal intermediate product lysosomal light ferritin (LLF). The siderosomal intermediate product was called siderosomal iron component (SIC). From CLF and LLF low-molecular-mass material was isolated by means of centrifugation at 95 000 *g*. The material was found in the supernatant, proved to be ferritin-related (see Fig. 6) and will be subject to further study.

Morphology

All products reacting with the anti-(rat liver ferritin) serum morphologically resembled ferritin: round particles lying in paracrystalline arrays as shown in the ESI micrographs of Fig. 3. The SIC, which indeed contained iron as shown by the EELS spectrum (Fig. 4), morphologically resembled haemosiderin (Fig. 3). Core size determination of ferritins is presented in Table 1.

Fe/P ratios

The Fe/P mass ratios of the ferritins, the SIC and the haemosiderin as determined by EPMA are presented in Table 1.

Iron determination

The amount of material available was just enough to calculate once only the number of iron atoms/ferritin molecule, indicating an iron-richer core in the lysosomal ferritins than in the cytosolic ferritins. Siderosomal ferritin-iron content could not be determined.

Protein analysis

On a 5% polyacrylamide gel CF, CLF, LF, LLF and SF each showed one band with the same migration rate staining positively for protein and iron. The material removed from CLF and LLF also showed one band, but this band migrated much faster and stained positively for protein but negative for iron. HS and SIC did not migrate at all.

On a 15% SDS gel CF, CLF, LF, LLF, SF, SIC and HS showed a band at 20.5 kDa. In addition to this band SIC and (sometimes) HS also showed several bands in the region of 60–67 kDa and a band at 36 kDa. HS (always) showed an extra band at 14 kDa (Fig. 5). The fast migrating material from CLF and LLF showed one band at 14.4 kDa (not shown).

The 20.5-kDa bands of CF, CLF, LF and LLF and the 14.4-kDa band of the fast migrating material from CLF and LLF were positive in the Western blot (Fig. 6). In the Western blot the 20.5-kDa subunit was found at

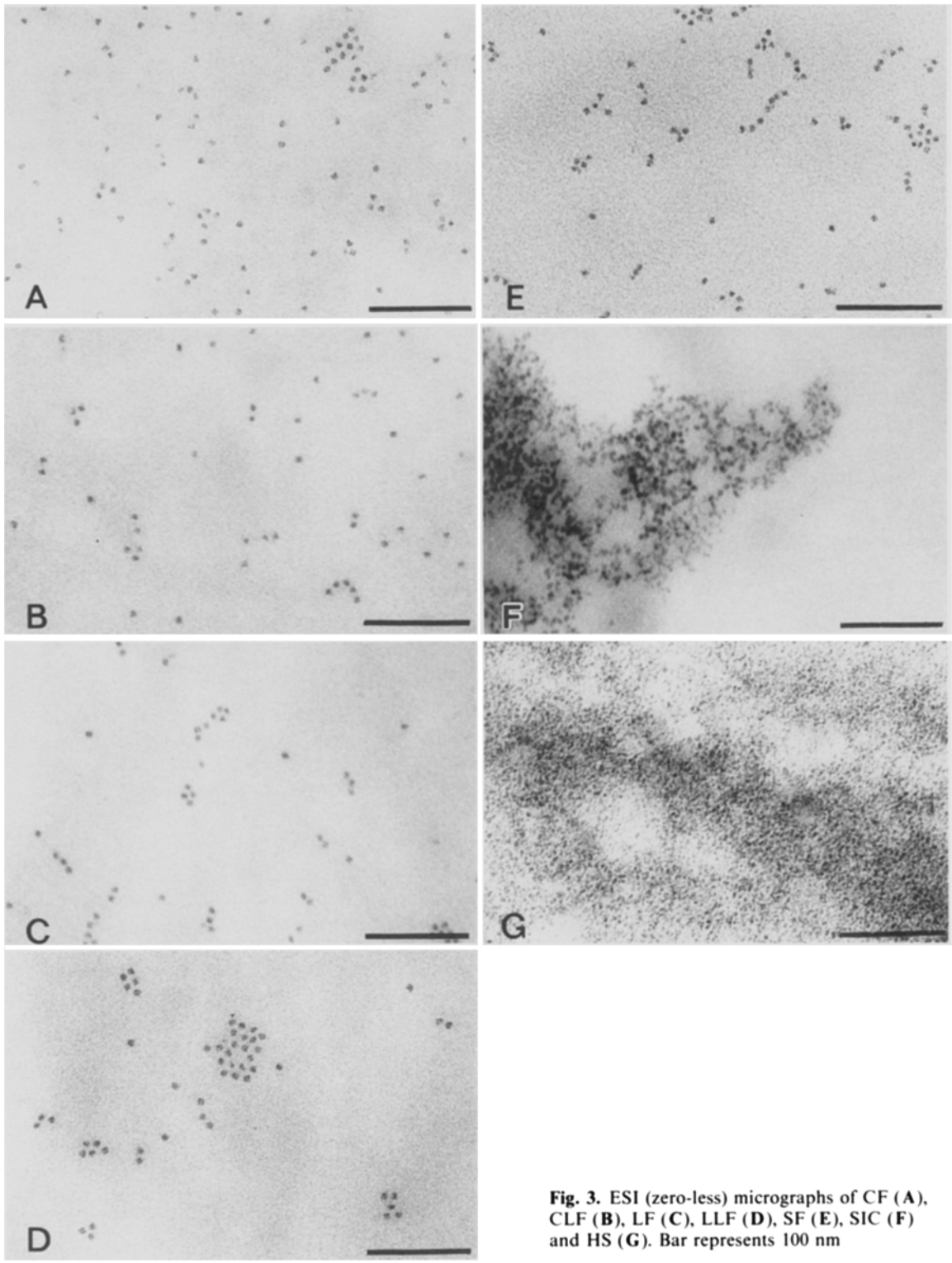

Fig. 3. ESI (zero-less) micrographs of CF (**A**), CLF (**B**), LF (**C**), LLF (**D**), SF (**E**), SIC (**F**) and HS (**G**). Bar represents 100 nm

20–23 kDa, depending on the marker used. Immunoblotting with siderosomal products was not possible due to unavailability of material. Therefore we used additional siderosomal material from fresh livers for the Western blot (Fig. 7). SF shows many extra smaller and larger peptides along with the 20.5-kDa peptide (43–67 kDa, 20–25 kDa and 12 kDa). SIC contains the 20.5-kDa peptide and a peptide of 18kDa. Haemosiderin reacts only faintly. CF and LF from fresh liver were used as a reference.

Discussion

In a previous study (Ringeling et al. 1989) to determine the effect of dialysis on Fe/P ratios of various rat liver

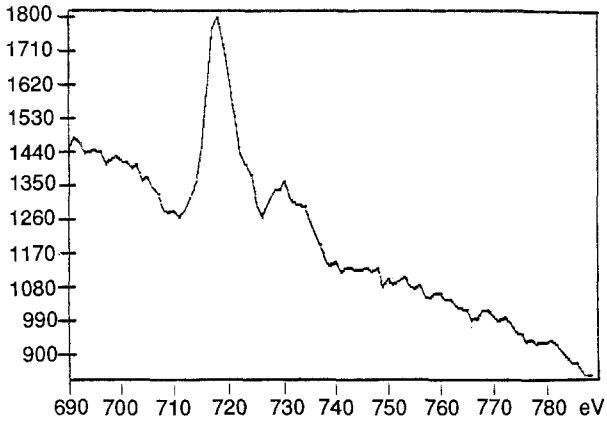

Fig. 4. EELS spectrum of SIC, with relative intensity on the *y* axis and energy (in eV) on the *x* axis

Table 1. Diameters of the iron cores and Fe/P ratios of all products

Fraction	*n*	Diameter (nm)	*P*	*n*	Fe/P	*P*
CLF	692	6.1±0.6		10	5.5±1.7	
CF	170	6.9±0.9	*	12	3.5±0.8	*
LLF	3074	6.7±0.8	*	10	4.9±1.1	*
LF	709	7.6±0.9	*	13	8.0±4.0	*
SF	544	7.2±0.9	*	13	10.6±3.6	n.s.
SIC	—	<6	*	10	12.1±2.8	n.s.
HS	—	<6	n.d.	12	17.5±3.4	*

Results are the average ±SD. * represents $P<0.05$ determined by the student *t*-test; n.s. = not significant; n.d. = not determined

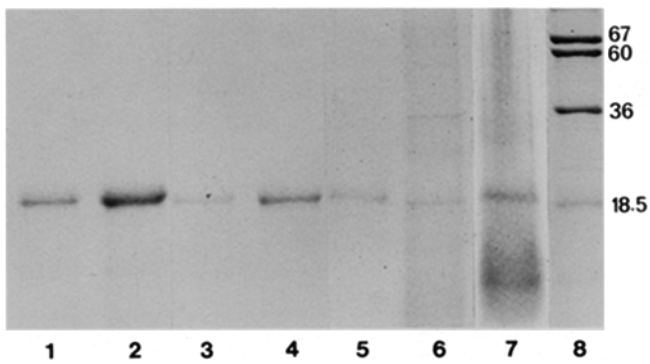

Fig. 5. SDS/PAGE of CF (1), CLF (2), LLF (3), LF (4), SF (5), SIC (6), HS (7), HMW Pharmacia (8)

ferritins, we obtained a cytosolic and a siderosomal ferritin fraction by Sepharose 6B chromatography. We mentioned that the main siderosomal fraction was very heterogeneous, containing in addition to ferritin large iron-deficient material. After chromatography over a Sepharose 4B column this fraction produced a fraction with an Fe/P ratio of 8:1 (unpublished). Moreover, the cytosolic fraction as used in the previous study displayed lysosomal enzyme activity, indicating contamination with lysosomal material. Considering these data, we decided to switch to a more gentle fractionation technique (Richter 1984) using the total liver homogenate before starting experiments with parenchymal cells vs sinusoidal cells. This technique allowed us not only to obtain a relatively pure cytosolic fraction, but also to separate the lighter (mainly ferritin-containing) lysosomes from the heavily loaded (mainly haemosiderin-containing) siderosomes. In this way we obtained, all in one procedure, (a) a cytosolic fraction, (b) a lysosomal fraction, (c) a siderosomal fraction and (d) haemosiderin (Fig. 1). From these fractions all iron-containing compounds were isolated (Fig. 2) and compared with each other. The low-molecular-mass material, probably representing partly degraded ferritin, was left out of the comparison because it does not contain the 20.5-kDa subunit but another subunit that requires further investigation.

Comparing the lysosomal products (LF and LLF, Fig. 2) with the cytosolic products (CF and CLF, Fig. 2) we see that they have much in common. The conclusion that the products from these cellular compartments are ferritins seems to be right, as they (a) react with anti-(liver ferritin antibodies), (b) are soluble in water, (c) contain a 20.5-kDa subunit reacting positively in the Western blot (Fig. 6), and (d) resemble ferritin morpho-

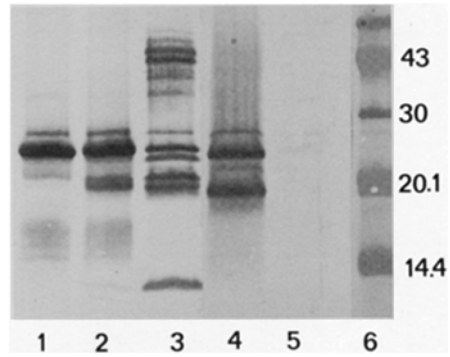

Fig. 6. Western blot after SDS/PAGE of LLF (1), LF (2), CLF (3), CF (4), low-molecular-mass material (5); LMW Pharmacia (6)

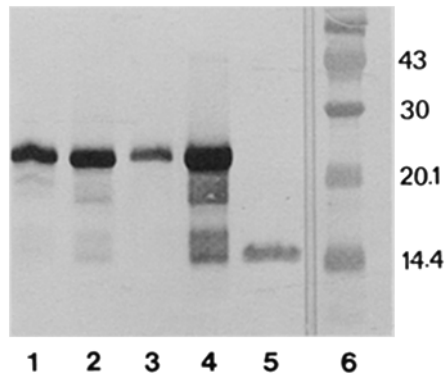

Fig. 7. Western blot after SDS/PAGE of additional material isolated from fresh livers. CF (1), LF (2), SF (3), SIC (4), HS (5); LMW Pharmacia (6)

logically (Fig. 3). However, the lysosomal products do differ from the cytosolic ferritins with respect to two parameters: iron-core size and Fe/P ratio, both increased in the lysosomal ferritins (Table 1). Our morphometric data about the core size are in line with those from Iancu et al. (1987) who observed a similar difference in size between cytosolic and lysosomal ferritin *in situ* in enterocytes from the small intestine of carbonyl-iron-loaded rats.

Preliminary biochemical results indicate that the iron content of our ferritins increases in parallel to the increase in iron core diameter. This would mean that lysosomal ferritin is still capable of taking up iron inside the lysosome. However, our biochemical data result from only one experiment. Besides, ferritin concentrations are calculated without taking into account the possible differences in immunological reactivity between cytosolic and lysosomal ferritins. Yet, when we combine our morphometric data, i.e. an increase in diameter and an increase in Fe/P ratio in the lysosomal ferritin, we must assume a (relative) increase in iron and/or a (relative) decrease in phosphorus. Protein analysis indicates that no changes occur to the protein coat after lysosomal uptake. Apparently, the first events that take place in the lysosomal compartment are the uptake and storage of ferritin without loss of its iron and without degradation of its mantle protein.

Comparing the siderosomal products with the lysosomal products, we see that they have less in common. Next to the siderosomal ferritin two other products are present. Unlike the siderosomal ferritin, these products, the siderosomal iron compound (SIC) and the haemosiderin, do not resemble ferritin greatly in that they are water-insoluble and that they do not react with specific anti-ferritin antibodies. It is not likely that the siderosomal iron compound is iron dextran, because intracellular iron dextran disappears from the liver over a two-week period following the iron-dextran injections (de Bakker 1983; Andrews et al. 1988b). All three siderosomal products differ from the lysosomal products in that their Fe/P ratio is increased and the iron core diameter is smaller. Assuming that siderosomal ferritin originates from lysosomal ferritin, the increase in Fe/P ratio with a simultaneous decrease in core diameter can only be explained by a heavy loss of phosphorus accompanied by or inducing a smaller loss of iron. This suggestion is supported by Treffry and Harrison (1978) who showed that phosphate is released more readily, and ahead of iron, from native ferritin. They suggested that much of the phosphate was located on core surface sites and therefore lost as surface iron is lost.

SDS/PAGE (Fig. 5) shows that all products are ferritin-related, as they all contain the 20.5-kDa subunit. In the Western blot, however, other ferritin-related peptides are also visible. These peptides are not present in the cytosolic ferritin, faintly in the lysosomal ferritin, but they are numerous in the siderosomal ferritin. As is known from literature (Crichton 1969) iron stabilizes the protein shell. A loss of iron, therefore, could lead to destabilization of the ferritin protein shell. Another possibility is that, as soon as iron is lost from the core, the ferritin protein coat is degraded by an iron-catalysed Fenton reaction. This reaction is supposed to occur in the siderosomes and to be responsible for lipid peroxidation of the siderosomal membranes, causing a higher fragility of these membranes (Bacon and Britton 1990; Selden et al. 1980). Both possibilities could explain why in the Western blot the 20.5-kDa subunit is found along with other peptides in the siderosomal products. Apparently, the siderosomal compartment is the place where the greatest changes to the ferritin molecule take place.

So, different processes take place in the lysosomes and in the siderosomes. The lysosomes function as a site where ferritin storage takes place, whereas the siderosomes function as a site of ferritin breakdown.

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