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IRON MOBILIZATION FROM CULTURED HEPATOCYTES: EFFECT OF DESFERRIOXAMINE B

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Abstract—When cultured rat hepatocytes prelabelled for different times at 37° with ^{59}Fe are reincubated for 1 hr in a fresh medium, radiolabelled iron is released in the washout medium as a function of the prelabelling time, and behaves like low molecular weight material on isokinetic centrifugation in sucrose gradients. When apotransferrin or desferrioxamine B are present in the reincubation medium, the kinetics of iron release are similar but the absolute amounts of radiolabelled iron found in the culture medium are much greater. In the presence of apotransferrin, most of the ^{59}Fe released from the cells distributes as transferrin whereas with desferrioxamine B, almost all the ^{59}Fe is extracted by benzyl alcohol indicating its chelation by the drug. Cell fractionation data indicate that iron accumulated by hepatocytes is rapidly incorporated into cytosol ferritin, and this seems to be a preferred source of iron for the chelator.

As reviewed by Bothwell *et al.* [1], the contribution of iron to many vital enzymatic reactions makes it essential for living organisms. While the total body iron for a man of 75 kg is about 5 g, only 1 mg of iron is absorbed and 1 mg is excreted every day. The conservative nature of iron metabolism conceals many exchanges of the metal between different body compartments. It is therefore easy to understand that the balance between iron absorption and excretion is critical implying several regulatory processes. Nonetheless, several hundred million people suffer from iron deficiency or overload.

At the cellular level, a model has been proposed [2–6] to explain transferrin iron uptake. It consists in the binding of transferrin to specific plasma membrane receptors, interiorization of the complex by receptor mediated endocytosis, release of iron in acidic granules, perhaps lysosomes, although a non-lysosomal acidic intracellular component has been recently proposed [7] and recycling of iron depleted transferrin back to the cell surface where it is released in the extracellular medium.

However, the mechanism of iron release from cells in normal physiological conditions, as well as the mobilization of cellular iron by chelators in iron overload remain poorly understood. While studies on parenchymal iron release from the perfused rat liver [8, 9] and from isolated hepatocytes [10] have been reported in the literature, the cellular mechanisms of iron mobilization remain at present unknown. Since iron overload concerns mainly the liver and in particular parenchymal cells, we have chosen cultured rat hepatocytes as an experimental model. We present here data on iron mobilization from ^{59}Fe loaded hepatocytes in the absence of chelators or in the presence of either apotransferrin or desferrioxamine B, an iron chelator widely used in the chemotherapy of human iron overload.

METHODS

Rat hepatocytes were isolated and cultured on collagen coated gas permeable Petri dishes (Petriperm dishes from Heraeus, G.F.R.) as previously described [11]. Transferrin was isolated from rat serum [3] and apotransferrin was obtained by dialysis against a sodium acetate buffer 0.05 M pH 5.5 containing 0.05 M EDTA. ^{59}Fe citrate with a molar ratio Fe to citrate 1 : 50 was from IRE (Fleurus, Belgium) and desferrioxamine B was from CIBA-GEIGY (Basle, Switzerland).

After incubation in 1 ml Hank's culture medium (Gibco Biocult, Paisley, Scotland) containing 1% bovine serum albumin (BSA) supplemented with 10 μM ^{59}Fe added either as ferric citrate or as saturated transferrin, the hepatocytes were washed successively 4 times with 2 ml of phosphate buffered saline (0.14 M NaCl, 3 mM KCL, 10 mM Na_2HPO_4/KH_2PO_4, pH 7.4 : PBS) once with 2 ml culture medium containing 10% foetal calf serum (Gibco) and twice with PBS. The cells were then either dissolved in 1% sodium deoxycholate adjusted to pH 11.3 with NaOH or reincubated for different durations in 1 ml of Hank's culture medium containing 1% BSA in the absence or the presence of desferrioxamine B or apotransferrin, and subsequently washed and dissolved as above. Aliquots of the incubation medium and cell lysate were assayed for radioactivity after dispersion in aqualuma cocktail (LUMAC Systems, Basle, Switzerland) in a liquid scintillation counter (PACKARD TRICARB CD 460, Packard Instruments, San Diego, CA, U.S.A.). The concentration of cell proteins was assayed according to [12], using BSA as a standard. The nature of the molecules with which ^{59}Fe was

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associated in the reincubation medium was analyzed by isokinetic centrifugation on sucrose gradients ranging from 5% (w/w) to 27% (w/w) [13]. After 64 hr centrifugation at 4° and 36,000 rpm in a SW41 rotor (BECKMAN, Palo Alto, CA, U.S.A.), the gradient was fractionated and 30 fractions were collected. The distribution of ^{59}Fe labelled compounds was compared to those of desferrioxamine B or transferrin used as standards.

For the cell fractionation experiments, hepatocytes incubated at 37° in the presence of ^{59}Fe citrate were washed four times with PBS, once with culture medium, twice with PBS and thereafter homogenized in 0.25 M sucrose supplemented with 3 mM imidazole pH 7.0, as previously described [11]. The homogenate was then separated into a nuclear (N) fraction and a cytoplasmic extract by 10 min centrifugation at 1700 rpm in a 259 rotor of an IEC PR 6000 centrifuge (DAMON, Needham, MA, U.S.A.) and washed in sucrose-imidazole. The cytoplasmic extract was further separated into a MLP fraction defined as in [14] and a final supernatant (S fraction) by a 30 min centrifugation at 40,000 rpm in a 50 Ti rotor (BECKMAN). The MLP fraction was further analyzed by isopycnic centrifugation on a linear density sucrose gradient ranging from 1.05 to 1.30 g/ml as previously described [11]. The distribution of iron was compared to those of 5'-nucleotidase and cathepsin B, marker enzymes respectively of plasma membrane [15] and lysosomes [16].

The molecular weight of the molecules with which the radiolabelled iron recovered in the S fraction was associated, was estimated by gel filtration on Ultrogel 3.4 (LKB, Villeneuve-la-Garenne, France). The elution profile of ^{59}Fe labelled molecules was compared to those of ferritin, transferrin or DNP lysine used as standards. The fractions obtained after isopycnic centrifugation were analyzed by polyacrylamide gel electrophoresis (from 7% to 30% (w/v) in acrylamide) in the presence of 0.1% sodium dodecyl sulfate (SDS–PAGE). The presence of radiolabelled iron was detected by autoradiography of the gels. On the other hand, the concentration of rat transferrin in the fractions obtained after isopycnic centrifugation was estimated by the particle counting immunoassay technique [17] using latex particles coated with anti-transferrin IgG obtained as previously described [3] and purified rat serum transferrin as standard. ^{59}Fe labelled ferrioxamine was extracted by benzyl alcohol as described by de Wael and Ploem [18].

RESULTS

Kinetic experiments

When cultured rat hepatocytes are incubated for 4 hr at 37° in the presence of 10 μM ^{59}Fe added in the extracellular medium either as ferric citrate or as saturated rat transferrin, they accumulate 860 and 76 picomoles of ^{59}Fe/mg of cell protein respectively. At this stage, when the cells are reincubated in a fresh medium, up to 40 picomoles of ^{59}Fe/mg of cell protein are released in the reincubation medium only if the cells have been loaded with ^{59}Fe citrate. However, in the presence of 50 μM desferrioxamine B in the reincubation medium, an additional amount of 40 pmoles of ^{59}Fe was mobilized whatever the source of iron. On the other hand, already after 10 min incubation in the presence of transferrin or citrate, more than 60% of the cell associated radiolabelled iron was bound to cellular ferritin as analyzed by gel filtration on Ultrogel 3–4. Furthermore, chelation experiments reported in the literature [19] using desferrioxamine show that the iron incorporated in cytosol ferritin is equally available for mobilization whether the cells have been initially labelled with iron from transferrin or nitriloacetate, another low molecular weight derivative of iron; therefore all of the loading experiments described in this paper were performed in the presence of 10 μM ^{59}Fe citrate.

As shown in Fig. 1, when cultured hepatocytes are preincubated for different times in the presence of 10 μM ^{59}Fe citrate, washed and reincubated for 1 hr in a fresh culture medium, part of the cell associated ^{59}Fe is released. The amount of radioactive iron found in the reincubation medium increases with the prelabelling time and after 4 hr, it represents 37.5 pmoles of ^{59}Fe/mg cell protein. As also shown in Fig. 1, the amounts of ^{59}Fe found in the reincubation medium increase in the presence of either 100 μg/ml rat apotransferrin or 50 μM desferrioxamine B; after 4 hr prelabelling, they respectively reach 60 and 86 pmoles of ^{59}Fe/mg cell protein.

When we consider the absolute amounts of ^{59}Fe mobilized by apotransferrin or desferrioxamine B, i.e. the difference between the radiolabelled iron found in the reincubation medium in the presence of these chelating agents and that found in their absence (Fig. 1), we can calculate that they increase with prelabelling time up to 20 min where a stable plateau is reached, which amounts to 20 pmoles (apotransferrin) and 40 pmoles (desferrioxamine B) of ^{59}Fe/mg of cell protein.

In another set of experiments, cultured hepatocytes were incubated for 20 min at 37° in the presence of 10 μM ^{59}Fe citrate and thereafter reincubated for

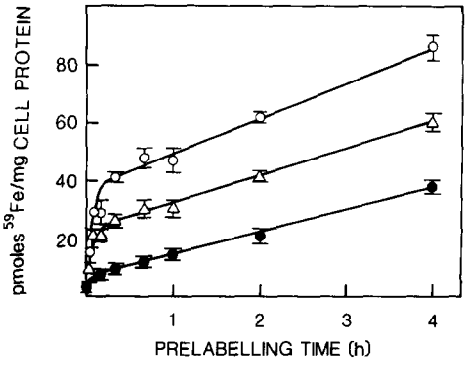

Fig. 1. Iron mobilization from ^{59}Fe preloaded cultured hepatocytes. Cells (about 2.5 mg protein) were incubated in 20 cm^2 collagen coated Petri dishes for different times at 37° in 1 ml of culture medium containing 10 μM ^{59}Fe citrate, washed and reincubated for 1 hr at 37° in a fresh medium in the absence (●) or the presence of 100 μg/ml apotransferrin (△) or 50 μM desferrioxamine B (○). At the end of the reincubation, the culture media were analyzed for the presence of radioactivity and the results correlated with the protein content of the hepatocytes. Mean results of 3 independent experiments ± S.D. are given.

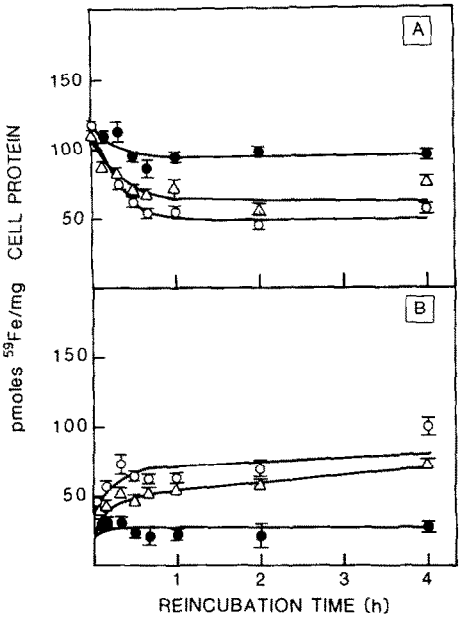

Fig. 2. Effect of reincubation time on the iron mobilization from ^{59}Fe labelled hepatocytes. Cells (about 2.5 mg protein) were incubated in 20 cm^2 collagen coated Petriperm dishes for 20 min at 37° in 1 ml of Hanks' medium in the absence (●) or the presence of 100 μg/ml of apotransferrin (△) or 50 μM desferrioxamine B (○). After the reincubation the cells were washed 4 times with PBS, once with culture medium and twice with PBS. The cells were then dissolved in sodium deoxycholate and analyzed for radioactivity and protein while the culture media were analyzed for the presence of radioactivity. (A) Amounts of ^{59}Fe which remain associated with the cells. (B) Amounts of ^{59}Fe released in the reincubation medium, correlated to the protein content of the hepatocytes. Mean results ± SD of 3 independent experiments are given.

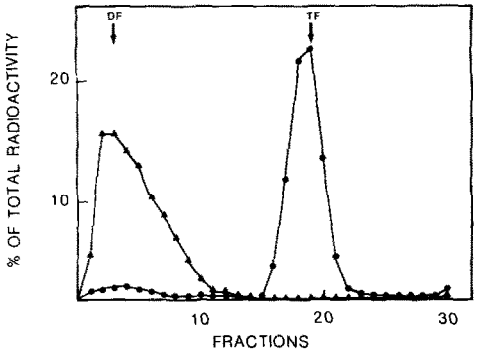

Fig. 3. Characterization of iron released from ^{59}Fe, preloaded cultured hepatocytes. Cells (about 2.5 mg protein) were incubated in 20 cm^2 collagen coated Petri dishes in 1 ml of culture medium containing 10 μM ^{59}Fe citrate, washed and reincubated in a fresh medium in the presence of 100 μg/ml of apotransferrin (●) or 50 μM desferrioxamine B (▲) as in Fig. 2. After the reincubation, the media were analyzed by isokinetic centrifugation on a sucrose density gradient ranging from 5% (w/w) to 27% (w/w). After 64 hr centrifugation at 4° and 36,000 rpm in a SW41 rotor, the gradient was fractionated and 30 fractions were collected. One of the gradients contained ^{59}Fe labelled desferrioxamine B (DF) and ^{3}H labelled transferrin (TF) as markers.

different times in a fresh medium. The results presented in Fig. 2 indicate that the amount of iron released from the cells in the culture medium increases at the beginning of the reincubation and rapidly attains a plateau. In the presence of apotransferrin or desferrioxamine B in the reincubation medium, the same kinetics are observed but the absolute amounts of iron released are greater.

The sedimentation coefficient of the molecules with which ^{59}Fe released in the culture medium was associated, was analyzed by isokinetic centrifugation on sucrose gradient. As indicated in Fig. 3, in the absence of apotransferrin or desferrioxamine B in the reincubation medium, the radiolabelled iron remains at the top of the gradient suggesting its association with low molecular weight molecules; in the presence of apotransferrin in the reincubation medium, all of the ^{59}Fe distributes like transferrin suggesting its association with the protein, whereas in the presence of desferrioxamine B ^{59}Fe remains at the top of the gradient and more than 80%, is extracted by benzyl alcohol, indicating its chelation by desferrioxamine B.

Subcellular localization of radiolabelled iron accumulated by the cells

The subcellular localization of ^{59}Fe accumulated by cultured hepatocytes was determined by cell fractionation. The apparent molecular weight of the molecules with which ^{59}Fe is associated was established by gel filtration or by autoradiography after SDS–PAGE. Hepatocytes were incubated for different times at 37° in the presence of 10 μM ^{59}Fe citrate, washed and homogenized in 0.25 M sucrose–3 mM imidazole, pH 7.0. The homogenate was then separated into N, MLP and S fractions by differential centrifugation. The distribution of iron between these 3 fractions does not change significantly as a function of the duration of the incubation: about 5% is recovered in N, 30% in MLP and 65% in S. Gel filtration of the S fraction (Fig. 4) indicated that after 5 min incubation, about 90% of iron distributes as ferritin, the rest as low molecular weight constituents; more than 85% of iron is recovered in the S fraction is also retained by antiferritin IgG immobilized on Sepharose protein A. After 1 hr incubation, ^{59}Fe recovered in the S fraction behaves almost exclusively as ferritin.

As shown in Fig. 5(A), after isopycnic centrifugation of an MLP fraction prepared from hepatocytes incubated for 4 hr at 37° in the presence of 10 μM ^{59}Fe citrate, the radiolabelled iron has a bimodal distribution with a major peak around densities of about 1.14 g/ml and another peak at densities around 1.17 g/ml. Autoradiography after SDS–PAGE of the fraction found around densities of 1.14 g/ml (Fig. 6) shows that ^{59}Fe distributes like ferritin used as standard. This ^{59}Fe labelled ferritin which is found around 1.14 g/ml after isopycnic centrifugation could represent either material enclosed in vehicles equilibrating at these densities or soluble ferritin which had migrated in the gradient to this position as a result of its high molecular weight and its high content in iron. To discriminate between these possibilities, flotation experiments were carried out. In this system, the MLP fraction is placed at the bottom

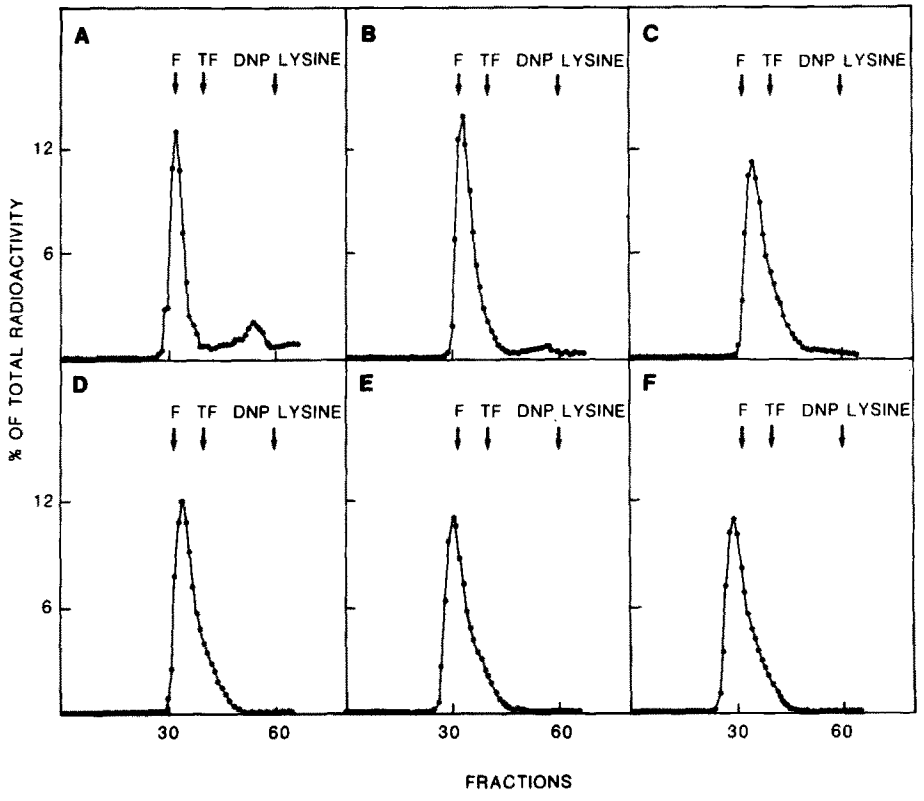

Fig. 4. Characterization of ^{59}Fe recovered in the soluble fraction of hepatocytes incubated for different durations at 37° in the presence of ^{59}Fe citrate. Cells (about 2.5 mg protein) were incubated in 20 cm^2 collagen coated Petri dishes for 5 min (a), 15 min (b), 30 min (c), 1 hr (d), 2 hr (e), 4 hr, (f) at 37° in 1 ml of culture medium containing 10 μM ^{59}Fe citrate. At the end of the incubation, the cells were washed and homogenized in 0.25 M sucrose supplemented with 3 mM imidazole pH 7.0. The nature of ^{59}Fe present in the S fraction was analyzed by gel filtration on Ultrogel 3.4. Ferritin (F), transferrin (TF), and DNP lysine were used as markers.

of the gradient so that organelles can move in the gradient to their equilibrium density while the soluble material remains at the bottom of the gradient. As shown in Fig. 5(B), iron recovered at the density of 1.14 g/ml is now found at the bottom of the tube demonstrating its association with cytosol ferritin. In contrast, iron recovered at the density of 1.17 g/ml in the experiment described at Fig. 5(A) now equilibrates at the same place which suggests its association with vesicles.

In order to try to characterize the iron which distributes at a density of 1.17 g/ml, SDS–PAGE was also performed. Unfortunately, the radioactivity recovered in the gel was too low to measure the presence of ^{59}Fe by autoradiography. Using immunological methods, we have measured that 0.34 μg of transferrin/mg of cell protein is associated with cultured hepatocytes and we have determined the distribution of transferrin after isopycnic centrifugation. The results presented in Fig. 5(A) indicate that transferrin distributes around a median density of 1.17 g/ml overlapping the distribution of iron which is not associated with ferritin. This suggests that at this density, iron and transferrin coexist in the same type of vesicle.

Effect of desferrioxamine B on the subcellular distribution of ^{59}Fe

To study the mechanism of iron chelation by desferrioxamine B, cultured rat hepatocytes were incubated for 20 min at 37° in the presence of 10 μM ^{59}Fe citrate, washed, and thereafter reincubated for 1 hr at 37° in a fresh medium in the absence or the presence of 50 μM desferrioxamine B. After the reincubation, the cells were washed, homogenized and fractionated. In the absence of desferrioxamine B in the reincubation medium, the distribution of iron associated with the MLP fraction is very similar to that observed in Fig. 5(A) with part of the ^{59}Fe distributed like ferritin and part like endogenous transferrin (Fig. 5C). In the presence of desferrioxamine B in the reincubation medium the repartition of iron between N, MLP and S fractions does not change significantly and the profile of iron recovered in the MLP fraction seems to be very similar to that observed in the absence of desferrioxamine B. However, the distribution of the cathepsin B, a marker enzyme of lysozomes, is shifted to lower densities. While in the absence of desferrioxamine B, less than 2% of ^{59}Fe distributing around 1.17 g/ml can be extracted by benzyl alcohol, we

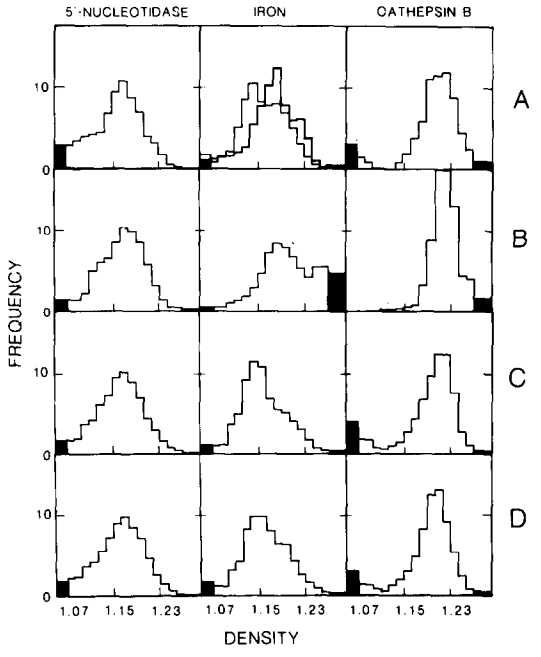

Fig. 5. Isopycnic centrifugation of MLP fractions. Hepatocytes incubated for 4 hr (a, b) or 20 min (c, d) at 37° in the presence of 10 μM ^{59}Fe citrate, washed and reincubated (c, d) or not (a, b) in the absence (c) or the presence of 50 μM desferrioxamine B (d) were fractionated. In (b) the sample was placed at the bottom of the gradient. The darker line in (a) represents the distribution of endogenous transferrin.

have established that, in presence of the chelator, from 40 to 70% of this ^{59}Fe is extracted, indicating its association with desferrioxamine B. These results suggest that while the major part of desferrioxamine B is excreted in the extracellular medium after chelation of iron, some remains associated with the MLP fraction and could be responsible for the observed change of lysosomal buoyancy. Furthermore, ^{59}Fe which distributes like endogenous transferrin, becomes partly extractable by benzyl alcohol. This could suggest that iron associated with transferrin can become a potential source of iron chelation for desferrioxamine B.

DISCUSSION

When cultured rat hepatocytes are incubated in the presence of 10 μM ^{59}Fe added either as ferric citrate or as saturated transferrin, they accumulate more iron from citrate than from transferrin, but when reincubated in the presence of desferrioxamine-B, the same amounts of iron are mobilized in the reincubation medium whatever the source of iron. The accumulation of ^{59}Fe citrate by hepatocytes probably result from an active transport system located in their plasma membrane [20]. Sixty-five percent of the radioactive iron taken up by hepatocytes seems to be very rapidly associated with cytosol ferritin since it is recovered in the S fraction, elutes like ferritin on Ultrogel 3.4 and is retained to more than 85% by anti-ferritin IgG immobilized on Sepharose protein A. However, our fractionation

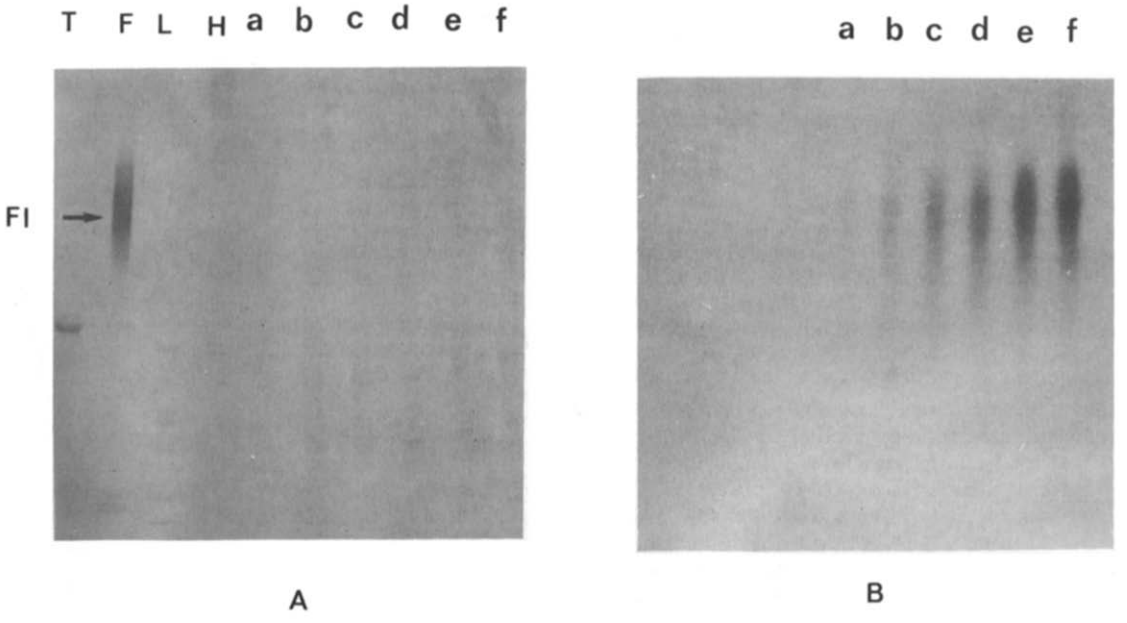

Fig. 6. Characterization of iron recovered in the MLP fraction of hepatocytes preloaded with ^{59}Fe citrate. After isopycnic centrifugation of MLP fractions from hepatocytes incubated for 15 min (a), 30 min (b), 1 hr (c), 2 hr (d), and 4 hr (e) at 37° in the presence of 10 μM ^{59}Fe citrate, the fractions recovered at densities around 1.14 g/ml were analyzed by SDS–PAGE. (a) The gel was washed and stained for proteins with Coomassie blue and for iron with 0.2% $K_4Fe(CN)_6$ (w/v) in 2% HCL. (b) ^{59}Fe recovered in the gel was analyzed by autoradiography. Transferrin (T), Ferritin (F), High molecular weight (H) and low molecular weight (L) products were used as standards. F.I.: Ferritin iron.

experiments indicate that 30% of ^{59}Fe is also recovered in the MLP fraction. Part of this radiolabelled iron behaves like ferritin as indicated by SDS–PAGE followed by autoradiography. Flotation experiments indicate that it consists of ferritin molecules which are either loosely adsorbed on membranes or represent a cytosol contamination of the MLP fraction. Another part of the ^{59}Fe associated with the MLP fraction equilibrates around 1.17 g/ml after isopycnic centrifugation in sucrose gradients. The overlapping of the distribution of ^{59}Fe and that of endogenous transferrin indicates that they could coexist in the same type of vesicles. Since cultured hepatocytes synthesize and secrete about 0.4 μg of transferrin per mg of cell protein and per hour [21], these vesicles could be implicated in the secretion of neosynthesized transferrin molecules into the culture medium.

When cultured hepatocytes are incubated for different time at 37° in the presence of ^{59}Fe citrate, washed and reincubated in a fresh medium, radiolabelled iron associated with low molecular weight molecules is released in the reincubation medium in amounts related to the prelabelling time. On the other hand, in the presence of desferrioxamine B and to a lesser degree apotransferrin in the reincubation medium, additional amounts of radiolabelled iron are released from the cells in a process related to the prelabelling time. Our kinetic studies clearly indicate that there is a compartment which is saturated in 20 min from which ^{59}Fe can be mobilized. We have tried to characterize this compartment.

The fact that in the presence of desferrioxamine B in the reincubation medium, 40–70% of iron recovered in the MLP fraction equilibrating at the same density as endogenous transferrin becomes extractable by benzyl alcohol, could suggest that transferrin is a potential source for desferrioxamine B. This supposes, however, that there is a mechanism allowing the dissociation of iron from the protein before its chelation by desferrioxamine B, since *in vitro*, at physiological pH, this chelator is unable to remove iron from transferrin [22]. The acidic intralysosomal pH could be responsible for that dissociation as we have proposed for fibroblasts [2–4] and erythroblasts [5]. However, considering the absolute amount of transferrin associated with the cells (0.34 μg/mg cell protein) and assuming that the protein is saturated with iron, transferrin can furnish only about 27% of iron which is mobilized by desferrioxamine B during a 1 hr reincubation of hepatocytes preloaded with ^{59}Fe. We must therefore conclude that there is another source of iron accessible to desferrioxamine B. Since already after 15 min incubation we do not observe significant amounts of iron associated with a low molecular weight product, the only possible other source of iron is ferritin. Since after a long prelabelling time, very small amounts of iron bound to ferritin seems to be released even after 72 hr of incubation in the presence of 50 μM desferrioxamine B, [23] we propose that after a short incubation time, iron associated with ferritin is not yet deeply incorporated within the shell of the protein, which could make it more accessible to the chelator. After longer incubation, the proportion of iron associated with ferritin but accessible to the chelator becomes very small as a result of the increasing incorporation into the ferritin iron micelle.

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