Biochemical Pharmacology, Vol. 38, No. 19, pp. 3233–3240, 1989.
Printed in Great Britain.

0006-2952/89 $3.00 + 0.00

DESFERRITHIOCIN AND DESFERRIOXAMINE B

CELLULAR PHARMACOLOGY AND STORAGE IRON MOBILIZATION*

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(*Received* 21 *December* 1988; *accepted* 31 *March* 1989)

Abstract—^{3}H-Desferrithiocin (DFT) has been synthesized from desmethyl desferrithiocin. The uptake and release of this ^{3}H siderophore and of its iron complex have been studied in cultured rat hepatocytes and systematically compared to ^{14}C desferrioxamine B (DFO). At 37°, the uptake of both chelators is strictly proportional to the extracellular concentration and no toxicity is observed up to, at least, 1 mM. Uptake of ^{3}H DFT is rapid and reaches a plateau after *ca.* 1 hr. The accumulation of ^{3}H DFT attains a maximum three times that of ^{14}C DFO and the plateau is reached much more rapidly. Upon reincubation in a drug-free medium of cells that had accumulated ^{3}H DFT, most of the ^{3}H label is rapidly released in the culture medium. These kinetic parameters suggest that the accumulation of these two chelators results from their diffusion across cellular membranes, as a function of the gradient of concentration between the cellular compartment and the extracellular medium. Differential centrifugation of homogenates from hepatocytes incubated with ^{3}H DFT shows that the bulk of cell associated ^{3}H-label (82%) is found in the cytosol, whereas a small proportion (14.5%) is present in the particulate fraction. Isopycnic centrifugation on sucrose gradients suggests that ^{3}H-label associated with the particulate fraction is localized within mitochondria. In contrast, ^{14}C DFO distributes in almost equal proportions between cytosol and the particulate fraction (MLP). At least part of the ^{14}C-label in MLP is associated with lysosomes. Rat hepatocytes cultivated for long term in synthetic culture medium have been used to study iron mobilization by chelators from ^{59}Fe loaded cells. DFT mobilizes iron more rapidly than DFO. This effect is also observed *in vitro* with ferritin, where, in addition, DFT is much more efficient than DFO to mobilize iron at acidic pH. These results strongly suggest that different iron mobilization from cultured hepatocytes results from differences in the cellular pharmacology of these two chelators and, in particular, in their rate of uptake, cellular accumulation levels and subcellular localizations. DFT could mobilize iron from cytosol and, possibly, to a small extent from mitochondria, whereas DFO would do so from cytosol and lysosomes.

Like other metals, iron is essential for living organisms. In man, its metabolism is highly conservative. Although there is a considerable daily exchange of iron between the various body compartments, its uptake and overall loss are very low, amounting to only *ca.* 1–1.5 mg per day [1]. The limited capacity to absorb dietary iron and to eliminate excess iron in man [2], results in some 500 million iron-deficient and several million people suffering from iron overload throughout the world.

Haemochromatosis develops when excessive iron is absorbed either from the gastrointestinal tract or from parenteral iron loading, as a result of repeated blood transfusions used, for example, in the treatment of β-thalassaemia [3]. Clinical manifestations of transfusional siderosis (liver alterations, tissue damage, heart failure, etc.) usually appear after 100 units of blood have been transfused [3, 4].

Effective therapy to remove iron is therefore imperative. The iron-chelating drug that has provided the best treatment is desferrioxamine B (DFO),† a microbial siderophore isolated from *Streptomyces pilosus* [5]. It is produced by large-scale fermentation under iron-limiting conditions and clinically characterized by low long-term toxicity. The selectivity of DFO for Fe(III) is excellent and its binding affinity for iron is much higher than that of transferrin. However high cost of production, short plasma half-life and the need of parenteral administration limits its potential value [6].

Over the past decades, several hundreds of molecules from synthetic, microbial or plant origin have been studied in cell culture and animal models. Some compounds have been identified and found to be superior to DFO in their chelating efficiency. A few of them are effective after oral administration [7].

Desferrithiocin (DFT) is a novel siderophore secreted by *Streptomyces antibioticus* DSM 1865 [8]. This compound is structurally unrelated to desferrioxamine and other well-known microbial siderophores. *In vitro*, it affects the growth of the malaria parasite *Plasmodium falciparum* at concentrations

* Supported by the Belgian FRSM (Grant No. 3.4551.88) and Ciba–Geigy, Basel, Switzerland.

† Abbreviations used: BDM-H, basal defined medium supplemented with hormones; DFO, desferrioxamine B methanesulphonate; DFT, desferrithiocin; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; FT, ferrithiocin; MLP, combination of the mitochondrial fraction (M), light mitochondrial (L) and microsomal (P) fraction; S, final supernatant.

Enzymes: cathepsin B (EC 3.4.22.1); cytochrome *c* oxidase (EC 1.9.3.1); esterase (EC 3.1.1.1); lactate dehydrogenase (EC 1.1.1.27); 5′-nucleotidase (EC 3.1.3.5).

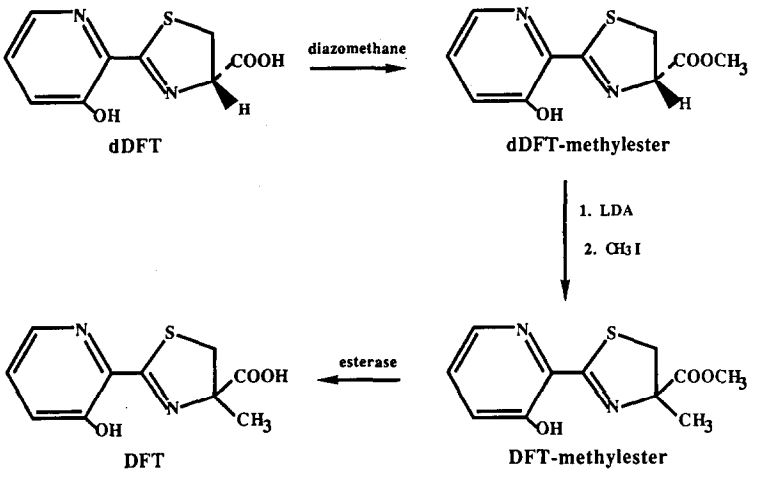

Fig. 1. Synthesis of desferrithiocin from des-methyl desferrithiocin.

comparable to DFO or human lactoferrin [9]. DFT is a small molecule with several different functional groups which complexes Fe(III) with a 2:1 stoichiometry [10]. Using an animal model, Longueville and Crichton [11] have demonstrated that DFT stimulates ferritin iron mobilization when administered either by gavage or by intraperitoneal injection. A preliminary pharmacokinetic study has shown that more than 50% of an oral dose is resorbed and excreted in the urine [10]. Using a model of cultured rat hepatocytes, Baker *et al.* [12] have observed that DFT is much more effective than DFO, both in reducing iron uptake and in mobilizing hepatocyte iron, but that it shows signs of toxicity at high concentrations. Toxicity has also been observed *in vivo* in the treatment of NMRI mice infected with *Plasmodium vinckei* [9].

Since iron storage and iron overload pathology concern mainly the liver and, in particular, parenchymal cells, cultured rat hepatocytes have been chosen as an experimental model [12, 13]. Laub *et al.* [14] have studied the uptake, release and subcellular distribution of two radiolabelled derivatives of DFO and their corresponding iron complexes in these cells and have proposed that ferritin iron mobilization by DFO and its derivatives occurs, at least in part, in lysosomes.

To characterize the sources of iron mobilized by DFT, we have synthesized ^{3}H DFT and studied its uptake and release in cultured rat hepatocytes, as a function of incubation time and chelator concentration. Its subcellular distribution was established by subcellular fractionation techniques. Results were systematically compared to those obtained with ^{14}C DFO, obtained from Ciba–Geigy (Basel, Switzerland). Iron mobilization by the chelators was also compared, *in vitro*, from long-term cultures in synthetic medium of ^{59}Fe loaded hepatocytes and from ferritin.

MATERIALS AND METHODS

Radiosynthesis of ^{3}H *DFT.* The synthesis of DFT from desmethyl DFT consists of a sequence of reactions of esterification, methylation and enzyme-catalysed hydrolysis, followed by titration with NaOH to form the water-soluble DFT sodium salt. The outline of the synthesis is shown in Fig. 1 (Peter H H, personal communication).

After esterification with diazomethane, 10 mCi of C_3H_3I (85 Ci/mmol, Amersham International, Amersham, U.K.) mixed with 0.6 ml CH_3I was added to 259 mg desmethyl DFT methylester. The product was thereafter hydrolysed enzymatically with porcine liver esterase (Boehringer, Mannheim, F.R.G.). The ^{3}H-label is recovered in the methyl group of DFT. The corresponding ferrithiocin (^{3}H FT) was prepared by mixing 0.1 mmol ^{3}H DFT Na salt with 0.05 mmol $FeCl_3$.

The ^{14}C desferrioxamine B methanesulphonate (DFO) was kindly supplied by Dr H. H. Peter of Ciba–Geigy Ltd (Basel, Switzerland).

Isolation and culture of rat hepatocytes. Rat hepatocytes were isolated and cultured, as previously described [14, 15] from male rats of the Wistar strain. They were cultivated on 20 cm^2 Petriperm, gas-permeable dishes (Heraeus, Danau, F.R.G.), coated with collagen in DMEM containing 10% (v/v) FCS (both from Gibco-Europe, Gent, Belgium). After 3–4 hr incubation in a water-saturated atmosphere under CO_2/air (7:93, v/v), the culture medium was changed and, for some experiments, replaced by a serum-free synthetic medium, consisting of basal defined culture medium [16] supplemented with insulin (10 μg/ml, Gibco), glucagon (100 ng/ml, Sigma, St Louis, MO), epidermal growth factor (50 ng/ml, Gibco), prolactin (20 mU/ml, Sigma), somatotropin (10 μU/ml, Sigma), linoleic acid/albumin (500 μg/ml, Sigma), trace element mixture (Gibco), ethanolamine (20 μM, Sigma), ascorbic acid (0.1 mM, Gibco), tocopherol (10 μM, Gibco) and dexamathasone (1 μM, Sigma) (BDM-H).

Hepatocytes cultured in this synthetic medium maintain their glucose-6-phosphatase activity, specifically take up and digest asialofetuin and secrete albumin and transferrin for at least one week (unpublished observations).

Kinetic experiments. Iron chelator accumulation

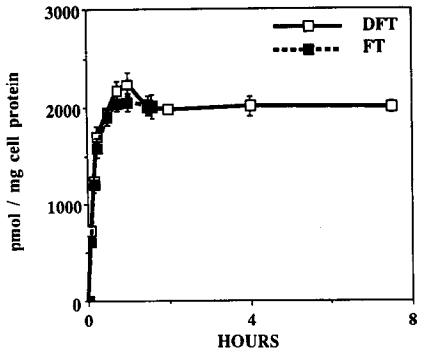

Fig. 2. Uptake of ^{3}H DFT or ^{3}H FT by cultured rat hepatocytes. Cells were incubated in 20 cm^2 collagen-coated Petri dishes in 1 ml culture medium in the presence of 500 μM ^{3}H DFT or ^{3}H FT at 37° for different durations. At the end of the incubation, they were washed twice with ice-cold PBS, once with culture medium and twice with PBS. The cells were then dissolved in 1% (w/v) sodium deoxycholate, pH 11.3 and analysed for radioactivity and protein whereas the culture medium was analysed for the presence of radioactivity. Mean of 3 independent experiments ± SD are given.

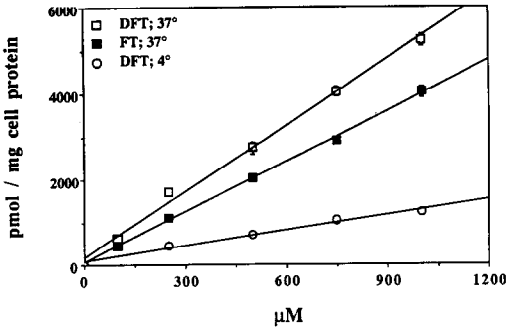

Fig. 3. Concentration dependence of the uptake of ^{3}H DFT or ^{3}H FT by cultured rat hepatocytes. Cells were incubated for 2 hr in the presence of different concentrations of ^{3}H DFT at 4° or 37° or of ^{3}H FT at 37°. At the end of the incubation, cells were washed, lysed and analysed as in Fig. 2. Mean of 3 independent experiments ± SD are given.

and release were measured exactly as described by Laub *et al.* [14]. After washings, cells were lysed in 1% (w/v) sodium deoxycholate adjusted to pH 11.3 with NaOH and analysed for protein content according to [17] using bovine serum albumin as standard.

The amount of ^{3}H label was determined after dispersion of 1-ml samples in 10 ml Aqualuma cocktail (Lumac, Basel, Switzerland) in a Tri-Carb 460 CD liquid scintillation system (Packard Instruments, San Diego, CA).

Subcellular fractionation. Experiments were carried out as previously described [14, 18]. In brief, cells were harvested in 0.25 M sucrose–3 mM imidazole, pH 7.3, homogenized by up and down strokes of the Dounce homogenizer (Kontess Glass Co., Vineland, NJ) and separated into the nuclear fraction (N) and the cytoplasmic extract by centrifugation (rotor 253, IEC CRU 5000 centrifuge, Damon, Needham, MA). The cytoplasmic extract was further separated into a particulate fraction (MLP) and a final supernatant (S fraction) by 30 min centrifugation at 40,000 rpm in a 50 Ti rotor (Beckman, Palo Alto, CA). The MLP fraction was then applied on top of a linear sucrose gradient ranging from 1.1–1.3 g/ml and centrifuged for 90 min in the VTi 50 rotor (Beckman) at 49,000 rpm. Fractions were collected and analysed; the results are presented according to Leighton *et al.* [19] in the form of normalized histograms. The distribution of radioactive material was compared to those of 5′-nucleotidase, cathepsin B and cytochrome *c* oxidase, marker enzymes respectively of plasma membrane, lysosomes and mitochondria [20–22].

Iron mobilization from hepatocytes. Cells were incubated for 24 hr in the presence of 10 μM ^{59}Fe-citrate (0.326 mCi/mmol, Ire-Celltarg, Fleurus, Belgium). After washing, cells were incubated at 37° in 2 ml BDM-H in the presence or absence of 50 μM DFO or 50 μM DFT. Every other day, the culture medium was renewed, the culture supernatants were collected and stored at −20° until assay of lactate dehydrogenase activity (Boehringer), radioactivity, albumin and transferrin secretion. At the end of the reincubation, the hepatocytes were washed, lysed in sodium deoxycholate and assayed for radioactivity and protein content as above.

The cytotoxicity of the chelators was assayed by measuring lactate dehydrogenase activity released in the culture supernatants.

Ferritin iron mobilization. Horse spleen ferritin (Boehringer) was diluted in buffers at different pHs to a final concentration of 100 μg/ml. After incubation for different times at 37° with 5 mM DFT or DFO, the amount of iron mobilized from ferritin was quantified by the measure of its extinction at 428 nm (DFO) and 430 nm (DFT).

RESULTS

Synthesis and analysis of ^{3}H *desferrithiocin*

The radiosynthesis of ^{3}H DFT consists of several reactions. To protect the carboxylic function of the desmethyl DFT from any chemical alteration during methylation, it was first transformed to its methyl-ester. The esterification by diazomethane takes place with quite good yield (80%).

After each reaction, the purity of the product was controlled by thin layer chromatography. The R_f (0.40) of ^{3}H DFT in CH_2Cl_2–CH_3OH–H_2O (70:25:4 v/v/v) is not significantly different from that of the unlabelled molecule. Specific radioactivity of about 1 Ci/mol was obtained.

The labelled product is a racemic mixture, as judged by polarimetric measure (1% w/v in ethyl alcohol; Perkin-Elmer 241 Polarimeter). The chemical structures were identified by ^{1}H–NMR (200 MHz in $CDCl_3$, Gemini-200, Varian) and IR spectroscopy (Perkin-Elmer 1710 Fourier Transform Infrared Spectrometer). Compared to the structural data for native DFT [8], the IR and NMR spectra of the synthetic ^{3}H DFT appear to be identical to those of native desferrithiocin.

Uptake and release of chelators

Figure 2 shows that at 37°, ^{3}H DFT and ^{3}H FT are

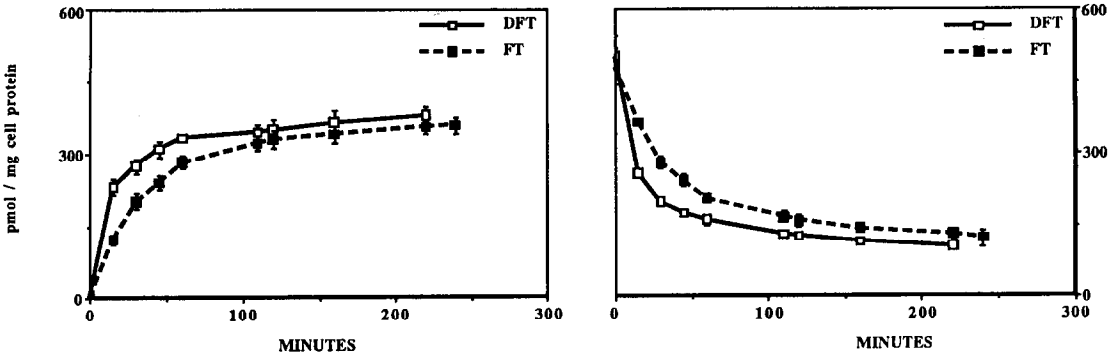

Fig. 4. Release of ^{3}H DFT or ^{3}H FT from hepatocytes. Cells were preincubated for 60 min at 37° in the presence of 100 μM ^{3}H DFT or ^{3}H FT. They were washed and reincubated in fresh medium for different durations at 37°. At the end of the reincubation, the amount of ^{3}H-label released into the medium (left) or remaining associated with the hepatocytes (right) were determined and correlated with cell protein content. Mean of 3 independent experiments ± SD are given.

rapidly accumulated by cultured rat hepatocytes on incubation in the presence of the drugs at 500 μM. The amount of cell-associated ^{3}H-label increases rapidly to attain a maximum after 1 hr and then levels off. About 60% of the cell associated ^{3}H label can be extracted by ethylacetate from cell lysates, suggesting that it is still associated with native DFT. The maximum intracellular concentration of the drug can be estimated to be respectively *ca.* 90% (DFT) and 82% (FT) of the concentration present in the culture medium at the beginning of the experiment, assuming a cellular volume of 5 μl/mg of cell protein [14, 23, 24]. When cultured rat hepatocytes were incubated at 37° in the presence of ^{14}C DFO, the amount of cell associated radioactive material reaches a plateau after 2–3-hr incubation (not illustrated).

In a second set of experiments, cultured rat hepatocytes were incubated for 2 hr at 37° or 4° in the presence of different concentrations of DFT or FT. Figure 3 shows that at extracellular concentrations ranging from 100 to 1000 μM, the amount of cell-associated radioactive material is strictly proportional to the external concentration. Upon incubation with ^{3}H FT, the amounts of cell-associated radiolabel represent about 77% of those reached in the presence of ^{3}H DFT. At 4°, the uptake of ^{3}H DFT is only 23% of that obtained at 37° but still remains proportional to the extracellular concentration. In this range of concentrations no toxic effects were observed by phase contrast microscopy (not illustrated).

At 37°, the uptake of ^{14}C DFO has the same characteristics as those of DFT, except that the accumulation levels are about 3 times lower than those reached with DFT (not illustrated).

The ^{3}H-label previously accumulated by hepatocytes preincubated at 37° in the presence of both 100 μM DFT or FT is quickly lost from the cells upon reincubation in drug-free medium at 37°. As shown in Fig. 4, the ^{3}H-label is rapidly released from the cells. After 4 hr reincubation, 22% (DFT) or 25% (FT) of the radioactive label initially accumulated remains associated with the cells.

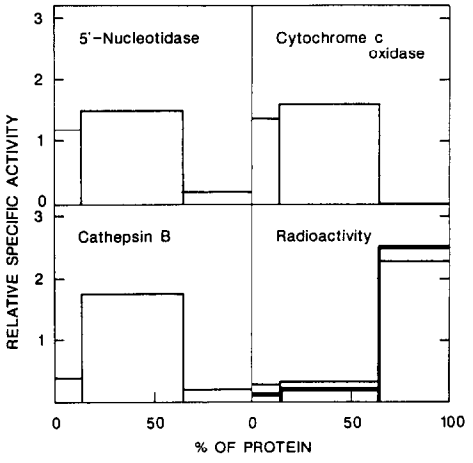

Fig. 5. Differential centrifugation in 3 fractions of homogenates from hepatocytes incubated with ^{3}H DFT. Cells were incubated for 1 hr (darker line) or 16 hr (normal line) at 37° in the presence of 500 μM ^{3}H DFT. After incubation, the cells were washed, homogenized and separated by differential centrifugation into 3 fractions as described in Materials and Methods. The fractions are respectively, from left to right, N, MLP and S. The distribution of the marker enzymes or constituents are the mean of 5 independent experiments.

Subcellular distribution of iron chelators

The subcellular distribution of ^{3}H label accumulated by cultured rat hepatocytes incubated in the presence of ^{3}H DFT was analysed by subcellular fractionation, after differential and isopycnic centrifugation.

After homogenization, cells were separated into a nuclear fraction (N), a MLP fraction (which is the combination of the mitochondrial fraction (M), light mitochondrial (L) and microsomal (P) fraction) and a final supernatant (S fraction). The distributions of radioactive labels and of marker enzymes are presented in Fig. 5. The bulk of ^{3}H label is recovered in the S fraction. Its percentage in the MLP fraction

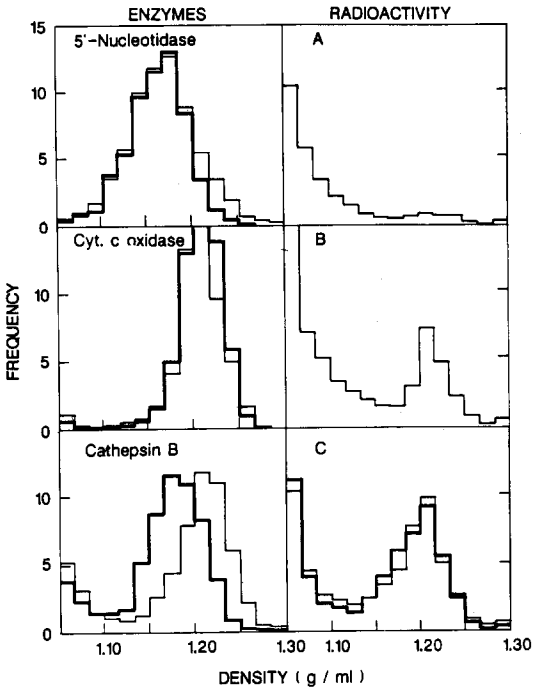

Fig. 6. Isopycnic centrifugation of MLP fractions prepared from homogenates of cultured hepatocytes. (A) ^{3}H DFT added to the MLP; (B) cells incubated for 1 hr with 500 μM ^{3}H DFT; (C) same as in (B) but for 16 hr. The darker line corresponds to cells incubated with 50 μM chloroquine for 1 hr. The lighter line represents cells incubated in the absence of chloroquine. Results are expressed as in [19]. The abscissa is the density span of the gradients divided in 15 sections of equal density increment; the ordinate is the frequency of constituents or activities in each section. The distributions of the marker enzymes or constituents are the mean of 5 independent experiments. Recoveries are respectively 94.9 ± 15.4% for radioactivity, 91.8 ± 10.6% for 5'-nucleotidase, 99.4 ± 26.8% for cytochrome *c* oxidase and 100.0 ± 18.0% for cathepsin B.

increases however from 9.5% after 1 hr incubation (darker line) to 14.5% after 16 hr incubation (normal line).

When chloroquine was present in the culture medium during the incubation, the distribution of lysosomal marker after differential centrifugation is affected: there is no significant changes in N fraction, but a large proportion of the cathepsin B activity initially present in the MLP fraction has passed into the S fraction (data not illustrated). In addition, 40% of the total cathepsin B activity is also found to be inhibited by chloroquine [25].

The MLP fractions from hepatocytes incubated with ^{3}H DFT for different times were further separated by isopycnic centrifugation on sucrose gradients. As indicated in Fig. 6, the marker enzymes are partially separated. When ^{3}H DFT is added to the MLP fraction of the homogenate from cells cultivated in a drug-free medium, the label present in the MLP fraction remains at the top of the gradient (Fig. 6A). When hepatocytes were incubated with 500 μM DFT for 1 hr, the distribution of the radioactive material present in the MLP fraction becomes

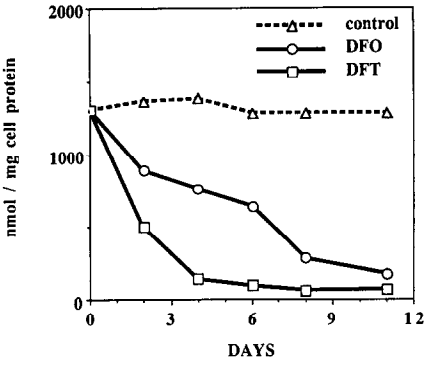

Fig. 7. Iron mobilization by chelators from ^{59}Fe preloaded rat hepatocytes. Cells were preincubated for 24 hr at 37° in 1 ml synthetic, BDM-H containing 10 μM ^{59}Fe citrate, washed and reincubated for different times in fresh medium in the absence or presence of 50 μM DFO or 50 μM DFT. The culture medium (2 ml/dish) was renewed every other day. At the end of the reincubation, cells were washed and dissolved in sodium deoxycholate; radioactivity was analysed and correlated with cell protein content.

bimodal (Fig. 6B): about two thirds of the ^{3}H-label are detected at the top of the gradient whereas one third equilibrates at densities around 1.21 g/ml, where the bulk of cytochrome *c* oxidase (mitochondria) and of cathepsin B (lysosomes) and a small proportion of 5'-nucleotidase (plasma membrane) are detected. When cells were incubated with the drug for 16 hr (Fig. 6C), about two-thirds of the radioactive label present in MLP fraction equilibrates at densities around 1.15–1.23 g/ml.

To further investigate the possibility that cell associated ^{3}H label could be associated with lysosomes and/or mitochondria, experiments were performed in the presence of chloroquine. This molecule accumulates in the lysosomes and decreases their buoyant density without affecting the density of the other organelles [18]. MLP fractions from hepatocytes treated with 50 μM chloroquine for 1 hr in the presence of ^{3}H DFT were fractionated on sucrose gradients. As illustrated by Fig. 6 (darker lines), the distribution of radioactivity in the MLP fraction from chloroquine-treated cells is not at all affected, whereas the distribution of cathepsin B is shifted to lower densities.

In vitro *mobilization of iron from hepatocytes by DFT and DFO*

After 24 hr incubation in the presence of 10 μM ^{59}Fe-citrate, about 1.3 nmol of radioiron is accumulated by the hepatocytes corresponding to 1 mg cell protein. Since each dish of 3×10^{6} hepatocytes has an average protein content of 3.85 mg (unpublished results), one may estimate that each cell has accumulated a mean of 10^{9} atoms of ^{59}Fe.

Upon reincubation of these cells in a culture medium containing no DFT nor DFO, no significant amount of ^{59}Fe is released into the culture medium (Fig. 7). In the presence of 50 μM of DFT or DFO, ^{59}Fe stored within the hepatocytes is rapidly mobilized. After 11 days reincubation, only 4.9% (DFT) and 13.4% (DFO) of the ^{59}Fe initially accumulated

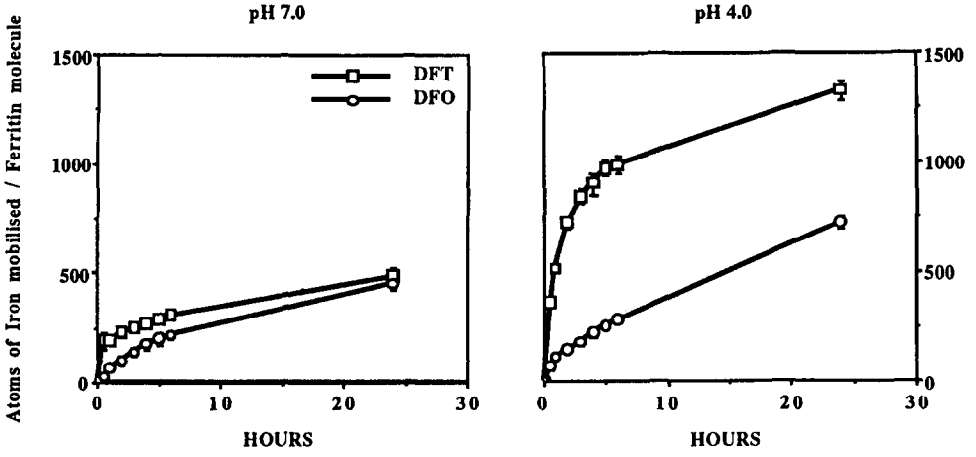

Fig. 8. Iron mobilization from ferritin by DFT and DFO. Ferritin (100 μg/ml) was incubated at 37° for different times with 1.0 M Mops buffer, pH 7.0 (left) or 1.0 M acetate buffer, pH 4.0 (right) supplemented with 5 mM DFT or DFO. The iron mobilized was followed spectrophotometrically and the results expressed as the number of iron atoms mobilized per ferritin molecule. Mean of 15 independent experiments ± SD are given.

remains associated with the cells. In addition however, these results indicate that although the total amounts of ^{59}Fe released from the hepatocytes are comparable at the end of the experiment, DFT mobilizes radioiron more rapidly than DFO.

In vitro *mobilization of iron from ferritin by DFT and DFO*

Figure 8 shows the mobilization, *in vitro*, of iron from ferritin by DFT and DFO after different times. At pH 4.0, the iron mobilization from ferritin by DFT is more rapid than by DFO (Fig. 8B). The difference between these two chelators in their iron mobilization ability is less important at neutral pH (Fig. 8A).

DISCUSSION

Uptake and release

Studies of the uptake and release of the radioactive iron chelators indicate that, at subtoxic doses, ^{3}H DFT and ^{14}C DFO are taken up by cultured rat hepatocytes in a concentration-dependent process (Fig. 3 and data not illustrated). After the same incubation time however, more ^{3}H label is accumulated by the cells than ^{14}C label. Comparable results were obtained in human lymphocytes, GM1380 cells and P388D1 cells using different experimental approaches (Vosbeck K, personal communication). By analogy with fibroblasts [23], assuming that 1 mg of cell protein represents a volume of *ca.* 5 μl [14, 24], the ratio between the intracellular and extracellular concentration, after 2 hr incubation at 37°, may be estimated to be, respectively, 1.04 for DFT and 0.3 for DFO. The accumulation level of the two chelators depends on the temperature and is decreased, upon incubation at 4°, to 23% (DFT) and to 37% (DFO) of the amount accumulated at 37°. These results support the hypothesis that the entry of DFT and DFO and, subsequently their release, consists in a diffusion controlled process by permeation across the plasma membrane.

At the same extracellular concentration of chelator, ^{3}H DFT is more rapidly taken up by hepatocytes than ^{14}C DFO (Fig. 2 and data not illustrated). This difference of diffusion rate could be explained by differences in the size, lipophilicity, structure, etc., of the two molecules. The molecular weights of DFO and DFT are respectively 657 and 260 and, whereas DFO is a linear molecule with three hydroxamate functions, DFT is formed by a conjugated heterocycle.

If ^{3}H DFT is previously complexed with Fe(III), its uptake by hepatocytes is decreased by 22.8% (Fig. 3). Since two molecules of DFT are needed to complex one atom of ferric iron, the size of the resulting complex (FT) is of course increased and this could explain a reduced rate of diffusion across the cellular membranes of the hepatocytes.

^{3}H DFT or FT accumulated by hepatocytes are rapidly released from cells upon reincubation in a drug-free medium, in a time-dependent process (Fig. 4), more rapid for DFT than for DFO. Only *ca.* 25% of ^{3}H label remains associated with the cells after 4 hr incubation. The release of DFT is more rapid than that of FT. Here again, the size of the molecules (DFT or FT) could explain the difference of the rate of release.

Subcellular distributions

Subcellular fractionation by differential centrifugation shows that most of the cell-associated ^{3}H-label accumulated during 1 hr incubation of the hepatocytes with ^{3}H DFT is recovered in the cytosol (S fraction). Analysis by thin layer chromatography followed by autoradiography of this fraction after 16 hr incubation suggests the presence of at least one labelled metabolite (results not shown). In addition, the particulate fraction (MLP) also contains ^{3}H-label in proportions increasing with the time of the incubation (Fig. 5).

When the MLP fraction from cells incubated with ^{3}H DFT was analysed by isopycnic centrifugation (Fig. 6), the ^{3}H-label distributed in two separate peaks. In view of the fact that ^{3}H DFT added to MLP is found at the top of the gradient, the first peak, could be due to diffusion or release of DFT from subcellular organelles during centrifugation. The second peak equilibrates at densities where mitochondria and lysosomes are also detected. When chloroquine is added to the culture medium in order to decrease the buoyant density of lysosomes [18], no detectable modification is observed in the distribution of ^{3}H-label, suggesting that the ^{3}H-label is associated with mitochondria. This hypothesis is reinforced by the observation that methylamine, a drug known to increase lysosomal pH [26] and to decrease intralysosomal concentration of drugs [27] has no effect on the uptake of DFT by hepatocytes (data not shown).

Subcellular fractionation of the hepatocytes incubated in the presence of ^{14}C DFO indicated that the bulk of the ^{14}C-label distributes as previously observed with ^{3}H methylated or acetylated derivatives of DFO [14] and that most of the label present in the MLP fraction can be associated with lysosomes (data not illustrated).

When cultured rat hepatocytes are incubated in the presence of ^{59}Fe added either as ferric citrate or as saturated transferrin, 65 to 85% of the accumulated radioiron appears rapidly associated with cytosol ferritin and this seems to be a preferred source of iron for the chelator [13 and unpublished observations]. In addition, both ferritin iron and ferritin protein are substantially increased in iron-overloaded animals [11]. The proportion of chelators found in the S fraction (82% for DFT and 53% for DFO) could explain the greater mobilization of iron either from cytosol ferritin [5, 28] or from the chelatable iron pool [28, 29].

In the case of methylated or acetylated derivatives of DFO which were found associated, for a part, with lysosomes, it was suggested that these molecules could mobilize iron from ferritin and/or haemosiderin present within lysosomes [14]. The results which confirm their subcellular localization with ^{14}C unmodified DFO strongly support this hypothesis. With DFT a small proportion of ^{3}H-label is present in the MLP fraction (14.5%) where it seems to be associated with mitochondria. It could correspond to DFT whose role is to chelate mitochondrial iron.

Iron mobilization

In vitro, the mobilization of ferritin iron by DFT and DFO proceeds in two phases (Fig. 8): the first, rapid, could result from chelation of iron already present in the 3-fold channels of the protein shell, as previously suggested [30]. The second phase could represent the mobilization of iron more slowly transferred from the interior of ferritin to external sites.

The rate of iron mobilization from ferritin is higher with DFT than with DFO and this is particularly important at acidic pH (Fig. 8B and unpublished results). In addition, if we consider that two DFT molecules are required to complex one iron atom, in comparison to the 1:1 stoichiometry of the DFO–Fe complex, the superiority of DFT for iron mobilization appears even more obvious. An higher efficiency of DFT than DFO to mobilize iron has also been observed in an animal model after intraperitoneal injection of chelators [11].

The observation that iron mobilization by DFO is more efficient at pH close to those prevailing within the lysosomes, combined with the cell fractionation results (see above) which indicate that ^{14}C DFO can be, for a large proportion, associated with these granules, strongly support the hypothesis that DFO is able to mobilize iron from ferritin (and/or from haemosiderin) present in lysosomes, as a result of cellular autophagy. In contrast however, the increased efficiency of DFT to mobilize ferritin iron at acid pH does not correlate with its subcellular localization, since cell fractionation results suggest that DFT does not accumulate within lysosomes.

In vitro, upon long-term reincubation of ^{59}Fe-loaded hepatocytes in culture conditions which preserve many cellular physiological functions, no significant amounts of ^{59}Fe are mobilized in the absence of DFO or DFT in the culture medium. In the presence of these chelators, only 5% (DFT) and 13% (DFO) of cell-accumulated ^{59}Fe remain associated with the hepatocytes after 11 days continuous exposure to the chelator (Fig. 7). Although the amounts of radioiron remaining in the cells are not very different at the end of the experiment, the rate at which DFT mobilizes the metal is higher than that of DFO.

These different rates could be explained by differences in the cellular pharmacology of the two drugs. First, at the same extracellular concentration, the amounts of DFT accumulated by the hepatocytes is about 3 times higher than that of DFO, although we have to remember that to complex iron, two molecules of DFT are required instead of one for DFO. On the other hand, subcellular localizations are also different since DFT is largely present in the cytosol (81.6%) and, to a lower extent, in the mitochondria (14.5%) whereas DFO is almost evenly distributed between lysosomes (42.1%) and cytosol (52.7%). This could result in the chelators having access to different intracellular iron chelatable pools: cytosol and, maybe, mitochondria for DFT; cytosol and lysosomes for DFO. In both cases however, iron chelators would enter the hepatocytes by diffusion across plasma membrane and chelate iron from these different sources. The resulting ferrithiocin and ferrioxamine complexes would thereafter get out of the cells also by diffusion, as a result of the concentration gradient between the intracellular and extracellular medium.

These results confirm previous studies [13, 14, 18] indicating that cultured rat hepatocytes provide a good model to study, *in vitro*, the efficiency of iron chelators to mobilize hepatic iron. Furthermore, the fact that these cells keep their biotransformation ability [18] combined with the use of synthetic serum-free culture medium to maintain physiologic properties over extended durations provides an interesting model to screen the efficiency of new iron chelators *in vitro*.

Acknowledgements—We thank Dr Ruth Laub for helpful discussion, Drs Etienne Sonveaux and Jacques Fastrez for

their help in the synthesis of desferrithiocin. The help of Feng Li for typing the manuscript is gratefully acknowledged.

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