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CELLULAR PHARMACOLOGY OF DEFERRIOXAMINE B AND DERIVATIVES IN CULTURED RAT HEPATOCYTES IN RELATION TO IRON MOBILIZATION

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Abstract—Two radiolabelled derivatives of deferrioxamine B (DF) have been synthesized: methyl-DF and acetyl-DF. Both derivatives are non cytotoxic and stable in cell culture but they are degraded in human plasma and more extensively in rat plasma.

Methyl-DF, acetyl-DF and DF mobilize radioiron to the same extent from hepatocytes loaded with ^{59}Fe citrate in the same range of extracellular concentrations. The uptake and release of the ^{3}H-labelled derivatives and their corresponding iron complexes have been measured and appear to represent a passive phenomenon resulting from the gradient of concentration between the cellular compartment and the extracellular medium.

The results indicate that only a limited pool of cellular iron is accessible for chelation and that neither the permeability of the cellular membrane, nor the intracellular concentration of the chelators are the limiting factors for iron mobilization. On the basis of the subcellular distribution of the ^{3}H-DF analogues, methylamine inhibition of iron chelation by siderophores in cell cultures and the positive effect of acidic pH and hydrolysis by lysosomal enzymes on *in vitro* iron mobilization from radiolabelled ferritin, we suggest that iron mobilization by DF and its derivatives occurs in lysosomes where they complex iron released from ferritin under the conjugate actions of acidic pH and lysosomal enzymes.

In man, iron metabolism is essentially conservative and iron losses are compensated by intestinal iron absorption. Because of the lack of a specific active excretion mechanism, iron overload occurs whenever excess of the metal enters the body either by increased gastro-intestinal absorption (as in idiopathic haemochromatosis) or as a result of repeated blood transfusions, used in the treatment of congenital anaemias such as β-thalassaemia. Clinical manifestations are described as considerably enlarged liver, cirrhosis, tissue damage (liver, pancreas, heart). Hepatoma and heart failure are the major causes of death [1, 2].

In 1964, Keberle indicated the advantages of the use of deferrioxamine B (DF),† a bacterial siderophore isolated from *Streptomyces pilosis* for the chemotherapeutic treatment of secondary iron storage diseases [3]. Deferrioxamine B is the most widely used iron chelator for clinical purposes: highly specific for Fe^{3+} (KS: 10^{31}), it ignores various other divalent cations (Ca^{2+}, Mn^{2+}, Cu^{2+}, Mg^{2+}, etc.) and is excreted in a non toxic form. Furthermore, DF inhibits collagen formation by fibroblasts and a reduction of liver fibrosis is observed in patients treated with DF [4]. Nevertheless, the source of iron mobilized by DF and its cellular target remains still largely unknown and controversial [5, 6]. Previous pharmacological studies on DF have been carried out both *in vitro* [7] and *in vivo* [8–10] by colorimetric detection of the chelator or after complexation of radioiron. In this way, DF metabolism by blood plasma and tissue slices could be demonstrated and the concentration of DF in biological fluids, blood and bile, after injection or perfusion could be measured.

Previous studies from this laboratory have shown that cultured rat hepatocytes loaded with ^{59}Fe citrate are an appropriate experimental model for studies on cellular iron mobilization [11].

To characterize further the interaction of DF with cultured hepatocytes at the cellular level, we have synthesized two radiolabelled derivatives of DF, ^{3}H-methyl-DF and ^{3}H-acetyl-DF. We have compared iron mobilization from ^{59}Fe-labelled hepatocytes by these derivatives with that of DF, and analysed the uptake and release of the ^{3}H-labelled DF analogues by the cells as a function of incubation time and chelator concentration. Their subcellular distribution was also established by cell fractionation techniques. Our results suggest that the labelled chelators accumulate within lysosomes. We therefore investigated the effect of pH on ferritin iron mobilization *in vitro* by DF and the influence of methylamine and ammonium chloride, two weak bases known to

* JNO is "Chargé de Recherche" of the Belgian FNRS.

† Abbreviations used: DF, deferrioxamine B; PBS, phosphate buffered saline (0.14 M NaCl, 3 mM KCl, 10 mM Na_2HPO_4-KH_2PO_4, pH 7.4); TNBS, trinitrobenzene sulfonic acid; N, nuclear fraction; MLP, combination of the mitochondrial fraction (M), light mitochondrial (L) and microsomal (P) fractions; S, final supernatant. Enzymes: Cathepsin B (EC 3.4.22.1); 5′-nucleotidase (EC 3.1.3.5); glycoprotein β D-galactosyltransferase (EC 1.9.3.1); cytochrome *c* oxidase (EC 3.1.3.9).

increase lysosomal pH [12, 13] and to impair the physiological function of lysosomes, on iron release from ^{59}Fe-labelled hepatocytes.

MATERIALS AND METHODS

Chemical methods. Reductive methylation (10–20 μmoles) of DF (Ciba Pharmaceuticals, Basle, Switzerland) was carried out in 1–2 ml of 0.1 M sodium borate buffer pH 9.0 in the presence of 4 equivalents of formaldehyde and 3 equivalents of sodium cyanoborohydride for 120 min at 20° [14]. The formation of methyl-DF was controlled by thin layer chromatography (polygram SilN-HR, Macherey-Nagel, Düren, F.R.G.) with ethanol : water : formic acid (v/v 80:15:15) as solvent. The products were detected by spraying aqueous 0.1 M $FeCl_3$. Further purification of methyl-DF was obtained by gel filtration on Sephadex LH20 (Pharmacia, Uppsala, Sweden) equilibrated and eluted with methanol.

Acetylation of DF (10–20 μmoles in 1–2 ml of 0.1 M sodium borate buffer pH 9.0) was performed with 2.2 equivalents of acetic anhydride for 120 min at 20° [15]. The formation of acetyl-DF was verified by thin layer chromatography (solvent : dichloromethane : methanol : water : formic acid v/v 82.47: 14.55:0.98:2.00). The spots were visualized with $FeCl_3$. Acetyl-DF was further purified on Dowex AG WX2 (Bio-Rad Laboratories, Richmond, CA), eluted by a gradient of ammonium acetate (pH 4.7) from 0.1 M to 2.0 M.

Radiolabelled methyl-DF and acetyl-DF were obtained by using sodium (^{3}H) cyanoborohydride (5–20 Ci/mmole) and (^{3}H) acetic anhydride (4–10 Ci/mmole, Radiochemical Centre, Amersham, U.K.). After 120 min incubation, an excess of unlabelled reagents was added. The ^{3}H-DF derivatives were purified by gel filtration on Biogel P2 (Bio-Rad Laboratories, Richmond, CA), eluted with 0.1 M ammonium carbonate pH 8.0 and lyophilized.

The corresponding ferrioxamines were prepared by mixing equal amounts of 0.1 M DF or its analogues with 0.4 M $FeCl_3$. The products were precipitated with ether, washed and dried. After dissolving in water, the ferrioxamines were purified by gel filtration on Biogel P2 as above.

Selective extraction of DF, methyl-DF, acetyl-DF, complexed or not with iron was performed by adding an equal volume of benzyl alcohol in the presence of 3.6 M sodium acetate. The concentrations of DF or its analogues were assayed by the method of Lowry [16] using the Folin reagent and 10 mM DF as standard. The response is linear in a range of concentration from 5 to 250 mM.

Free amino-groups were measured with the trinitrobenzene sulphonic acid reagent (TNBS) [17]. They were also detected on silicagel sheets by the ninhydrin reagent (Merck, Darmstadt, F.R.G.). Spectral determination of DF or its analogues was performed with a Beckman DU-7 spectrophotometer.

The stability of ^{3}H-DF analogues was tested by benzyl alcohol extraction after overnight incubation at 37° in the presence of plasma, serum or purified lysosomal enzymes [18].

Biological methods. Rat hepatocytes were isolated and cultured on collagen-coated, gas permeable, hydrophobic Petri dishes (20 cm^2) (Heraeus, Danau, F.R.G.) in 3 ml of Eagle–Dulbecco medium containing 10% foetal calf serum (Gibco-Biocult, Paisley, Scotland) as previously described [19]. The mean cell protein content per dish was about 3 mg.

The cytotoxicity of the chelators and the weak bases was assayed by measuring lactate dehydrogenase activity [20] in culture media or by Trypan blue exclusion.

Kinetic experiments were adapted from [11]. In summary, hepatocytes were incubated at 37° with 1 ml Eagle–Dulbecco medium containing 10 μM Fe citrate (22 mCi/mmole, IRE, Fleurus, Belgium) or ^{3}H-DF compounds. At the end of the experiment, the supernatant was collected and the cells were washed at 4°, twice with phosphate buffered saline (PBS), once with culture medium containing 10% foetal calf serum, twice with PBS. The cells were then lysed in 1% (w/v) sodium deoxycholate adjusted to pH 11.3 and analyzed for protein content [16] using bovine serum albumin as standard. The amount of radioactive material in cell lysates and culture medium was determined after dispersion in Aqualuma cocktail (Lumac Systems, Basle, Switzerland) in a Beckman LS 7800 counter (Palo Alto, CA).

For the cell fractionation experiments, hepatocytes were washed, homogenized in chilled 0.25 M sucrose supplemented with 3 mM imidazole pH 7.0, and separated into a nuclear (N), a particulate fraction (MLP) and a final supernatant (S) as in [19]. The MLP fraction was further fractionated by isopycnic centrifugation on a linear sucrose density gradient ranging from 1.05 g/ml to 1.30 g/ml as in [19]. The distribution of ^{3}H-label was compared to that of 5′-nucleotidase, cathepsin B and cytochrome c oxidase, marker enzymes respectively of plasma membrane, lysosomes and mitochondria [21–23] and the results are presented in the form of normalized histograms [24].

^{59}Fe-ferritin was partially purified from the S fraction of cultured hepatocytes loaded with ^{59}Fe citrate at 37° by gel filtration on Ultrogel 3.4 (LKB, Villeneuve-la-Garenne, France) eluted with PBS. ^{59}Fe-ferritin eluted as single peak and 89% of this material was precipitated by anti-ferritin IgG raised in rabbit (in contrast with 2% by control IgG). ^{59}Fe-ferritin was also purified by a cycle of high-speed centrifugations [25]. Its specific activity was about 0.8 μCi of ^{59}Fe/OD_{280}. The *in vitro* effects of pH and lysosomal extracts were performed as follows: ^{59}Fe-ferritin (1.6 nCi) was incubated in the presence of 5 mM cysteine, 0.01% sodium azide and 50 mM buffer (sodium acetate or sodium potassium phosphate) at the desired pH, for different times at 37°. The effects of lysosomal enzymes were measured in the presence of 20 μg protein/ml of rat liver lysosomal enzymes purified as in [18]. After various incubation times, an aliquot of 100 μl was taken, free iron was extracted by benzyl alcohol in the presence of 100 μM DF and the radioiron content of the organic phase was measured. Simultaneously, a sample of the incubation mixture at pH 4.8 was analysed by chromatography on Ultrogel 3.4 with 0.1 M sodium acetate buffer pH 4.8 as eluant.

RESULTS

Synthesis and purification of DF derivatives

The R_f of methyl-DF, acetyl-DF and DF are respectively 0.42, 0.83 and 0.83 with ethanol–water–formic acid as solvent and 0.09, 0.40 and 0.07 with dichloromethane–methanol–water–formic acid as solvent. Reactions with TNBS or ninhydrin reveal that for the DF derivatives, 70%–100% of the free amino groups have been masked. Purification of methyl-DF or acetyl-DF from DF can be achieved by gel filtration respectively on Sephadex LH 20 or Dowex AG WX2 (not shown). Labelling of these DF analogues enables specific activities from 60 to 140 mCi/mM for methyl-DF and from 40 to 410 mCi/mM for acetyl-DF to be obtained. These modifications of the chelator do not significantly change the maximum of their absorption spectrum at 228–237 nm (not shown).

Stability of ^{3}H chelators and cytotoxicity

Most of the ^{3}H-methyl-DF and ^{3}H-acetyl-DF are extracted by benzyl alcohol after 17 hr incubation at 37° in the presence of cultured hepatocytes, purified rat liver lysosomal enzymes at pH 4.8, and rat serum (Table 1). After 17 hr incubation in the presence of rat plasma, only 10% of ^{3}H-methyl-DF and 15% of ^{3}H-acetyl-DF are extracted by benzyl alcohol. Using the same conditions of incubation in the presence of human plasma, the ^{3}H-label recovery by extraction is 57% (methyl-DF) and 83% (acetyl-DF). DF analogues are not cytotoxic for cultured hepatocytes after overnight incubation at 37° at concentration up to 400 μM, as indicated by the absence of release of lactate dehydrogenase activity in the culture medium (not shown).

Iron mobilization by DF or its derivatives

Cultured hepatocytes, preloaded with 10 μM ^{59}Fe citrate for 20 min at 37°, were reincubated for various times in a fresh medium supplement with 50 μM methyl-DF, acetyl-DF or DF. As illustrated in Fig. 1, all three chelators mobilize radioiron from hepatocytes. Acetyl-DF seemed to be the most effective but this difference did not appear to be significant in further studies. The presence of cycloheximide (1 mg/ml) in the culture medium does not modify the kinetics of iron release nor the amounts of radioiron liberated (data not shown).

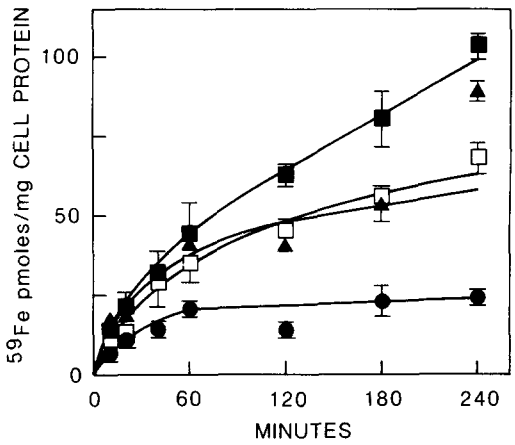

Fig. 1. ^{59}Fe release from hepatocytes in the absence (●) or in the presence of 50 μM DF (▲), methyl-DF (□) or acetyl-DF (■). Hepatocytes were preincubated for 20 min with 10 μM ^{59}Fe-citrate. The cells were washed and reincubated for different durations in fresh medium containing DF or its analogues. At the end of the incubation, culture medium was collected and the cells were lysed. Radioactivity and protein content were determined. Mean of two independent experiments ± S.D. are given.

Hepatocytes preloaded for 20 min with ^{59}Fe citrate, were reincubated for 60 min with DF, methyl-DF or acetyl-DF at concentrations ranging from 10 to 1000 μM. A minimum of 100 μM for

Table 1. Stability of ^{3}H chelators incubated for 17 hr at 37° in various experimental conditions

Experimental conditions of incubation	pH	Chelator concentration (μM)	Percent of ^{3}H-label extracted by benzyl alcohol	
			^{3}H-methyl-DF	^{3}H-acetyl-DF
PBS	7.2	5	92 ± 6	106 ± 9
Sodium acetate buffer (10 mM)	4.5	5	98 ± 3	96 ± 1
Lysosomal enzymes* (3.8 mg protein/ml)	4.5	5	108 ± 9	90 ± 7
Cultured rat hepatocytes*	7.2	10	73 ± 3	77 ± 3
Rat serum† (90%)	7.2	5	81 ± 12	79 ± 5
Rat plasma†				
9%	7.2	5	24	21
90%	7.2	5	24	16
Human plasma†				
9%	7.2	5	92	107
90%	7.2	5	57	83

* ^{3}H-methyl-DF and ^{3}H-acetyl-DF were incubated for 17 hr at 37°. The incubation media and the cells were then extracted with benzyl alcohol. Results are expressed as the percentage of ^{3}H-label present at the beginning of the experiment recovered in the organic phase.

† Freshly prepared serum or plasma, diluted in PBS in the presence of 0.01% sodium azide. Mean of two independent experiments ± S.D. are given except for incubation with plasma.

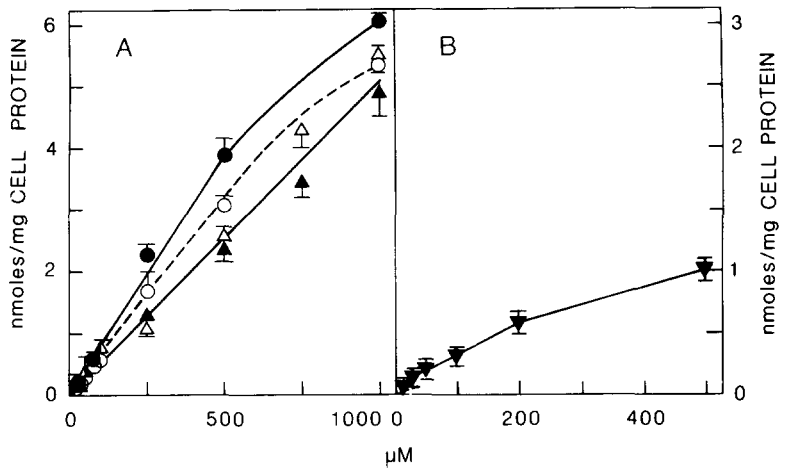

Fig. 2. Uptake of ^{3}H chelators by cultured hepatocytes. Cells were incubated for 60 min at 37° in the presence of different concentrations (A) of ^{3}H-methyl-DF (▲), ^{3}H-acetyl-DF (●) or ^{3}H-methyl-ferrioxamine (▼) (B). The cells were then washed, lysed and radioactivity determined and correlated with cell protein content. The ^{3}H-label was also extracted with benzyl alcohol from cell lysates: (△) ^{3}H-methyl-DF and (○) ^{3}H-acetyl-DF.

each type of chelator in the extracellular medium is required to obtain a plateau of iron mobilization (results not shown). The mean of 16 experiments using DF, methyl-DF or acetyl-DF allows us to calculate a mean release of 96 ± 42 pmoles of ^{59}Fe/mg cell protein after 1 hr of mobilization. Subtracting the release of ^{59}Fe into the culture medium from cells incubated in the absence of chelator, this value is 47 ± 27 pmoles ^{59}Fe/mg cell protein which represents 17% of the radioiron uptake (280 ± 76 pmoles/mg cell protein). No significant difference is detected between the three chelators. Only 6%–26% of the cell-associated radioiron is extracted with benzyl alcohol whereas more than 80% of the ^{59}Fe released into the culture medium is recovered in the organic phase.

Prolongation of the mobilization time in the presence of DF or its derivatives, results in an increase of ^{59}Fe release which amounts to 96 ± 28 pmoles/mg cell protein after 4 hr and 136 ± 18 pmoles/mg cell protein after 17 hr of treatment by the chelators (spontaneous iron release subtracted).

Uptake and release of ^{3}H chelators complexed or not with iron

^{3}H-methyl-DF and ^{3}H-acetyl-DF at a concentration of 100 μM are taken up by cultured hepatocytes and accumulation levels reach a plateau after 60 min incubation. Twenty min preincubation of the cells with iron citrate does not modify the uptake kinetics (results not shown). Figure 2A shows that after 60 min incubation, the uptake of tritiated methyl-DF or acetyl-DF is proportional to their concentration in the culture medium from 10 to 1000 μM. More than 80% of the cellular ^{3}H-label is still extracted by benzyl alcohol. If at that time, the cells are washed and reincubated in fresh medium, the ^{3}H-label is rapidly released into the culture medium in a form which can be extracted by benzyl alcohol (Fig. 3). After 4 hr incubation, 38% (methyl-DF) to 18% (acetyl-DF) of the ^{3}H-label initially accumulated by the hepatocytes, remains associated with the cells.

The uptake and release of ^{3}H chelators complexed with iron by hepatocytes has also been studied. Figure 2B indicates that ^{3}H-methyl-ferrioxamine is taken up by hepatocytes as a function of its external concentration. For a concentration of 100 μM in the

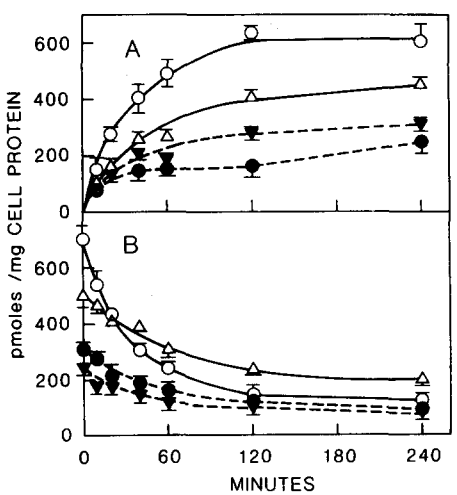

Fig. 3. Release of ^{3}H chelators from hepatocytes. Cells were preincubated for 60 min in the presence of 100 μM ^{3}H chelators. The cells were washed and reincubated in fresh medium for different durations. At the end of the incubation, the amount of ^{3}H-label released into the medium (A) or remaining in the cells (B) were determined and correlated with cell protein content. (△) ^{3}H-methyl-DF, (○) ^{3}H-acetyl-DF, (▼) methyl-ferrioxamine, (●) acetyl-ferrioxamine. Mean of two independent experiments ± S.D. are given.

Table 2. Distribution of marker enzymes and ^{3}H chelators between subcellular fractions

Enzymes	Percent in fractions		
	N	MLP	S
5′-Nucleotidase	12.2 ± 4.9	71.7 ± 6.3	16.0 ± 10.0
Cathepsin B	2.9 ± 1.2	83.2 ± 3.3	13.9 ± 2.8
Cytochrome c oxidase	9.5 ± 9.6	90.5 ± 9.6	n.d.*
Protein	7.7 ± 2.3	50.7 ± 7.8	41.6 ± 8.4
^{3}H-methyl-DF			
30 min	3.1	22.4	74.5
60 min	2.4	24.4	73.2
1000 min	2.6	37.6	59.8
^{3}H-acetyl-DF			
30 min	2.9	20.7	76.4
60 min	2.5	25.2	72.3
1000 min	5.8	33.8	60.4

Hepatocytes incubated for different times in the presence of 100 μM ^{3}H-methyl-DF or ^{3}H-acetyl-DF were homogenized and separated into nuclear (N) and particulate (MLP) fractions and final supernatant (S).
* n.d. not detectable.

culture medium, the amount of ^{3}H-methyl-ferrioxamine and ^{3}H-acetyl-ferrioxamine accumulated by the cells are respectively 0.28 ± 0.02 nmoles/mg cell protein and 0.31 ± 0.02 nmoles/mg cell protein, which represents only half of the internal concentration of ^{3}H-methyl-DF and ^{3}H-acetyl-DF (respectively 0.56 ± 0.17 nmoles and 0.64 ± 0.20 nmoles per mg cell protein) accumulated by the hepatocytes in the same conditions. If cells loaded with ^{3}H-ferrioxamine analogues are reincubated in fresh medium, the ferrioxamine compounds are rapidly released into the culture medium and after 4 hr reincubation, only 31–35% of the ^{3}H-labels initially accumulated remains associated with the cells (Fig. 3).

Intracellular distribution of cell-associated ^{3}H chelators

Hepatocytes, incubated in the presence of ^{3}H-methyl-DF or ^{3}H-acetyl-DF for different times at 37°, were homogenized and separated into a nuclear fraction (N), a MLP fraction and a final supernate (S fraction) (Table 2). The percentage of different marker enzymes and of ^{3}H-label in these three fractions is presented in Table 2. The distribution of the ^{3}H-label between these subcellular fractions is similar for ^{3}H-methyl-DF and ^{3}H-acetyl-DF. After a short incubation time (30–60 min), most of the cell-associated ^{3}H-label is found in the cytosol fraction (72–76%), whereas after 1000 min the content of the S fraction decreases to 60%, 34–38% being associated with the MLP fraction.

After isopycnic sucrose gradient ultracentrifugation of the MLP fraction, most of the ^{3}H-label captured during 30–60 min incubation equilibrates at the same densities as 5′-nucleotidase, a marker enzyme of the plasma membrane and as cathepsin B, a marker enzyme of lysosomes. However, after 1000 min, the distribution of both ^{3}H chelators becomes largely similar to that of cathepsin B, suggesting their association with lysosomes (Fig. 4.).

Effects of methylamine on iron mobilization

In order to study a possible functional role of lysosomes in iron mobilization by DF, ^{59}Fe-loaded hepatocytes were preincubated for 30 min in the presence of 10 mM methylamine and then reincubated in fresh medium containing 100 μM DF or methyl-DF and methylamine for different times. Figure 5A shows that the amount of radioiron mobilized by DF or methyl-DF is reduced respectively by 40% and 33% after 4 hr of treatment with methylamine. The uptake of ^{3}H-methyl-DF by hepatocytes

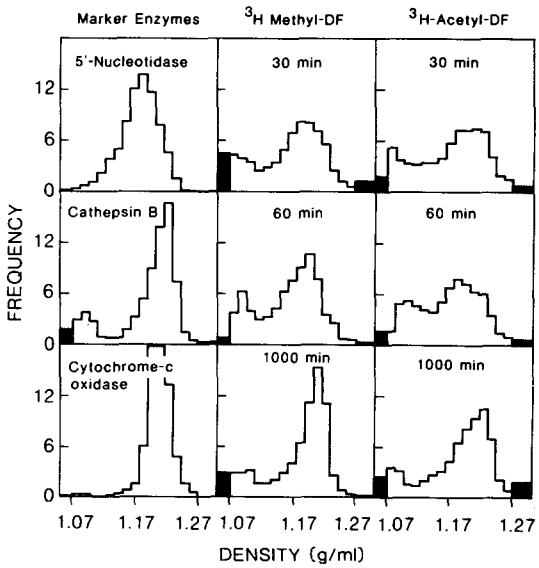

Fig. 4. Isopycnic centrifugation in linear sucrose gradient of MLP fractions prepared from cultured hepatocytes. The cells are cultured for different times (30 min to 1000 min) in the presence of 100 μM ^{3}H-methyl-DF or ^{3}H-acetyl-DF. The abscissa is the density span of the gradients divided in 15 sections of equal density increment; the ordinate is the frequency of constituents or activities in each section.

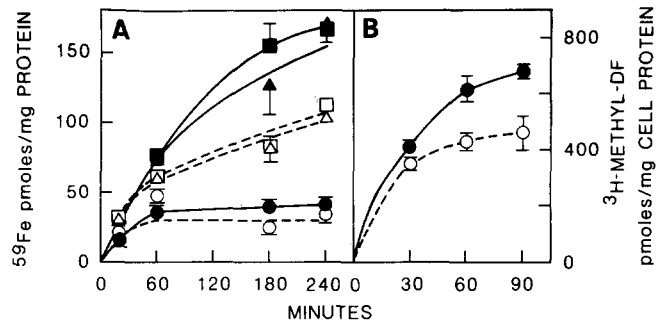

Fig. 5. Effect of methylamine on iron mobilization and chelator uptake by hepatocytes. (A) Hepatocytes which had been labelled with ^{59}Fe citrate, preincubated for 30 min at 37° in the presence (○, △, □) or the absence (●, ▲, ■) of 10 mM methylamine, were reincubated for different durations in fresh medium containing 100 μM DF (△, ▲) or methyl-DF (□, ■) and 10 mM methylamine (△, □). At the end of the reincubation, the cells were washed and radioactivity was measured in the wash-out medium and correlated with the cellular protein content. (B) Uptake of ^{3}H-methyl-DF in the presence of methylamine by hepatocytes. Cells were incubated with 100 μM ^{3}H-methyl-DF in the presence (○) or absence (●) of 10 mM methylamine. At the end of the incubation, the cells were washed and lysed. Radioactivity was determined and correlated with cellular protein content. The mean of three independent experiments ± S.D. are given.

is also reduced by 31% after 90 min incubation in the presence of methylamine (Fig. 5B). Similar results are obtained with ammonium chloride (results not shown).

In vitro *mobilization from ferritin*

^{59}Fe ferritin was purified from cultured ^{59}Fe-loaded hepatocytes and incubated at various pH values (from pH 4.0 to pH 7.4) for different periods of time at 37°. Figure 6B shows that after 3 hr incubation at pH 4.0 or 4.8, radioiron is released from the storage protein into the incubation medium whereas no mobilization occurs at pH 6.2 and 7.2. This release is proportional to the duration of the incubation and is not significantly influenced by the presence of 10 mM methylamine in the medium (Fig. 6). After 64 hr at 37°, 57% of the radioiron content of ferritin is recovered in the medium buffered at pH 4.0 whereas at neutral pH only 14% of the radioiron is released. The addition of DF to the medium enhanced the liberation of radioiron from the protein: 85% of the ferritin radioiron can be extracted by benzyl alcohol after 64 hr incubation at acidic pH. This increase in iron mobilization is lost when the pH of the experimental medium was raised to 6.2. A further addition of purified lysosomal

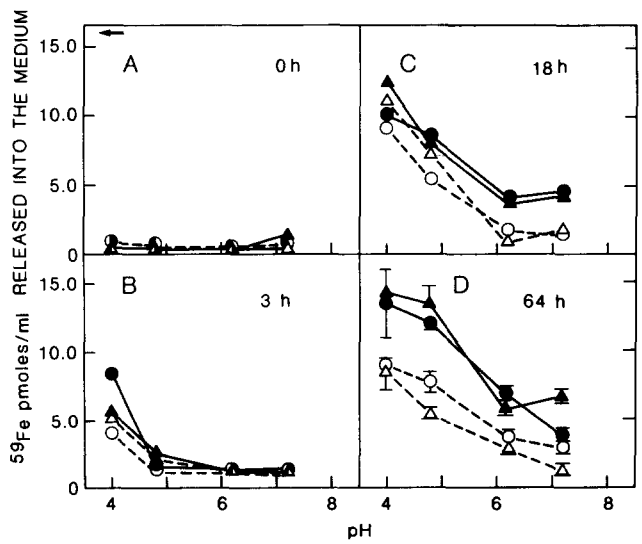

Fig. 6. Effect of pH on iron mobilization from ferritin by DF. ^{59}Fe-ferritin (1.2 nmoles ^{59}Fe/OD_{280}) was partially purified from cultured hepatocytes loaded 20 min with 10 μM ^{59}Fe citrate. The ^{59}Fe-labelled protein (16 pmoles ^{59}Fe/ml) was incubated at 37° at various pH in the presence (●, ▲) or absence (○, △) of 10 μM DF, and 10 mM methylamine (△, ▲) for different durations: 0 hr (A), 3 hr (B), 18 hr (C) and 64 hr (D). The amount of ferritin radioiron is indicated by the arrow (←). At the end of the incubation, radioiron was extracted in the presence of benzyl alcohol after adding 100 μM DF. Mean of two independent experiments ± S.D. are given.

enzymes to the reactional mixture (pH 4.8) at the beginning of the incubation magnified iron mobilization by a factor of 1.2–1.4.

DISCUSSION

Synthesis and stability of DF analogues

DF, the water soluble mesylate of deferrioxamine B, is a linear molecule with three hydroxamic groups in the interior which can complex Fe^{3+} [26] and one free amino group. To protect these hydroxamic groups from any chemical alteration during synthesis of DF derivatives, different authors complexed these molecules transiently with Fe^{3+}, but an additional treatment with strong acids or alkalis must be introduced to remove iron from its tight complex [27]. Working at pH 9, in mild conditions, we have synthesized methylated and acetylated derivatives of DF (Fig. 7). The acetyl and methyl groups are linked to the free amino group of the molecule as demonstrated by the absence of reaction with a specific reagent for free amine function such as ninhydrin and TNBS. Chromatographic properties of methyl-DF and acetyl-DF differ from DF and allow us to purify them in good yield. It is worth noting that natural derivatives of DF are synthesized by species of actinomycetes (Streptomyces, Nocardia, etc.) and are quite good siderophores [28, 29]. Three natural DF-derivatives with an amino-group are known: D_1, D_2 and E. D_1 may be similar to our acetylated product: their R_f on thin layer are similar in both chromatographic systems. The stability of methyl-DF and acetyl-DF was tested in various biological systems using as criterion, their extraction after overnight incubation at 37° by benzyl alcohol. Incubation of the DF derivatives in the presence of cultured hepatocytes, purified rat liver lysosomal enzymes or rat serum reduces extraction maximally by 27%, in contrast to the drastic loss of solubility in the organic solvent after incubation in the presence of rat or human plasma (up to 76%). The metabolic transformations of DF in plasma were first described by Meyer-Brunot *et al.* who suspected an α2-globulin to be responsible for DF hydrolysis [7].

Iron mobilization by DF and its derivatives

Previous studies [11, 30] have demonstrated that iron chelation is similar whether the cells have been initially labelled with iron derived from transferrin or from Fe-citrate. Methylation or acetylation of DF does not significantly alter its capacity to mobilize iron from iron-loaded hepatocytes. Iron mobilization is maximal at extracellular concentrations of the siderophores of 100 μM which represents the plasma concentration attained in clinical treatment [31] and represents, after 60 min incubation, 17% of the radioiron initially accumulated by the hepatocytes and 49% after overnight treatment. These results agree with clinical observations which underline the effects of a continuous exposure of a chelatable iron pool to a steady state level of DF [32, 33].

Uptake and release of ^{3}H chelators

Tritiated methyl-DF and acetyl-DF are taken up by hepatocytes or macrophages [34]. By analogy with fibroblasts [35], assuming that 1 mg of cell protein represents a volume of 5 μl, we calculate that after 60 min, the intracellular concentration of these derivatives are respectively 1.12 and 1.28 times the concentration present in the culture medium. This is sufficient to complex all of the radioiron initially taken up by the hepatocytes. Our data suggest that the entry of methyl-DF and acetyl-DF, and subsequently their release, are essentially diffusion controlled (by permeation across cellular membranes) in contrast with bacteria where this process is receptor-mediated [36]. If ^{3}H chelators are complexed with non-radioactive iron, their uptake by hepatocytes is reduced by 50%. However, their release from the cells is time dependent like the uncomplexed forms. Only 20–30% of the initially accumulated ^{3}H-ferrioxamine derivatives remains inside the cells. These results agree with the cellular radioiron content, extractable by benzyl alcohol at the end of iron mobilization experiments.

The intracellular concentrations of ^{3}H chelators and the permeability of the cell membrane to ferrioxamine complexes are not the limiting factors for iron mobilization and the extent of iron chelation depends on an intracellular radioiron pool. These results confirm similar observations carried out on Chang cells [37], hepatocytes in suspension [38], perfused liver [31], as well as *in vivo* studies on rats [32] and humans [10] which suggested the concept of a "transient iron chelatable pool" [40, 41], susceptible to be complexed by hydroxamic chelators.

Subcellular distribution of ^{3}H chelators

In a relatively short period of time after loading

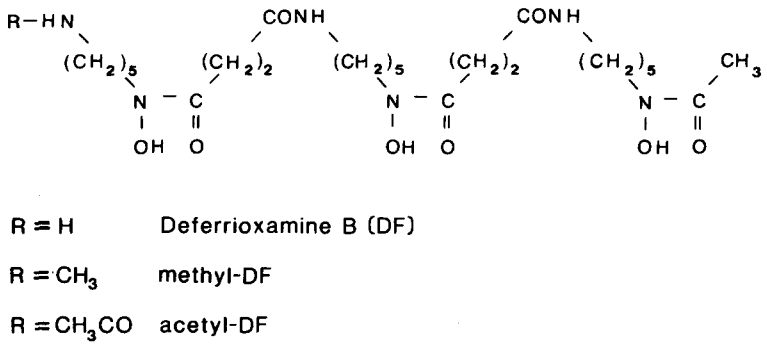

Fig. 7. Deferrioxamine B and its analogues.

hepatocytes either with iron chelates or with transferrin, iron-loaded ferritin appears in the cytosol and in lysosomes [42]. In cells, DF chelates iron at the expense of ferritin, not directly but from an iron chelatable pool [31, 44]. *In vitro* it is able to remove iron from ferritin at a slow rate ([3, 43, 44]—see also Fig. 6). Previous work in this laboratory failed to demonstrate a low molecular weight iron pool in hepatocytes and showed that the radioiron is rapidly incorporated into ferritin. Our present results point out some new considerations. The cell fractionation experiments indicate that the ^{3}H-labelled chelators are accumulated progressively as a function of time into subcellular organelles (most probably plasma membrane-related structures and lysosomes). After a long term incubation, isopycnic centrifugation indicates that the ^{3}H-label equilibrates at the same densities as lysosomes although some association with mitochondria cannot be rule out.

Possible role of lysosomes in iron mobilization by DF and analogues

In addition to a possible association of ^{3}H-labelled DF analogues with lysosomes, our results further indicate that when the intralysosomal pH is raised from 4.8 to 6.2 in the presence of 10 mM methylamine [13], iron mobilization by DF or methyl-DF diminished 30–40%, although the relative stability of DF for Fe^{3+} is higher at neutral pH (K_{eff}:10^{17}) than at pH 4.8 (K_{eff}:10^{14}) [45]. In the same conditions, ^{3}H-methyl-DF uptake is reduced by 30%. *In vitro*, iron mobilization by the ^{3}H chelator from ^{59}Fe-ferritin is 3–10 times more important at pH 4.8 (intralysosomal pH) than at neutral pH. Similar observations were reported by Wagstaff *et al.* [46] on human spleen ferritin in the unphysiological presence of dithionite. The pH effect is enhanced in the presence of purified lysosomal enzymes. Morgan, in a recent report, showed that bipyridine and phenanthroline (Fe^{2+} chelators) chelate iron in membrane fractions, probably endosomes characterized by a low pH [47] whereas under haem synthesis inhibition conditions iron is mobilized in cytosol and mitochondria [48]. At the present moment, we do not know if DF chelates iron in another subcellular compartment than lysosomes.

CONCLUSIONS

In conclusion, on basis of our results, we propose tentatively the following mechanism to explain iron mobilization by DF: after endocytosis of ferritin, haptoglobin–haemoglobin or haem–haemopexin [9, 43, 49] or autophagy of cytosolic ferritin, the synthesis of which is stimulated in the presence of a source of iron, iron would be released within lysosomes due to the conjugate action of acidic pH and hydrolytic enzymes. DF or its derivatives taken up by the cells and accumulated within lysosomes would chelate the released iron. Ferrioxamine complexes would then be secreted in the extracellular medium. In the absence of chelator, iron or natural iron complexes would pass through the lysosomal membrane, and gain access to mitochondria or to the cytosol where it could assure metabolic activities or be stored inside ferritin molecules.

The implications of our results are clear in view of the fact that quantitatively the largest pool of iron potentially available for chelation in iron-loaded individuals is intralysosomal ferritin and haemosiderin [50–52].

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