Determination of the chelatable iron pool of isolated rat hepatocytes by digital fluorescence microscopy using the fluorescent probe,Phen-Green SK

ARTICLE *in* HEPATOLOGY · APRIL 1999

Impact Factor: 11.06 · DOI: 10.1002/hep.510290435 · Source: PubMed

3 AUTHORS, INCLUDING:

Frank Petrat
Bayer
54 PUBLICATIONS **1,513** CITATIONS
SEE PROFILE

Ursula Rauen
University Hospital Essen
142 PUBLICATIONS **3,656** CITATIONS
SEE PROFILE

Determination of the Chelatable Iron Pool of Isolated Rat Hepatocytes by Digital Fluorescence Microscopy Using the Fluorescent Probe, Phen Green SK

FRANK PETRAT, URSULA RAUEN, AND HERBERT DE GROOT

The intracellular pool of chelatable iron is considered to be a decisive pathogenetic factor for various kinds of cell injury. We therefore set about establishing a method of detecting chelatable iron in isolated hepatocytes based on digital fluorescence microscopy. The fluorescence of hepatocytes loaded with the fluorescent metal indicators, phen green SK (PG SK), phen green FL (PG FL), calcein, or fluorescein desferrioxamine (FL-DFO), was quenched when iron was added to the cells in a membrane-permeable form. It increased when cellular chelatable iron available to the probe was experimentally decreased by an excess of various membrane-permeable transition metal chelators. The quenching by means of the ferrous ammonium sulfate + citrate complex and also the "dequenching" using 2,2′-dipyridyl (2,2′-DPD) were largest for PG. We therefore optimized the conditions for its use in hepatocytes and tested the influence of possible confounding factors. An *ex situ* calibration method was set up to determine the chelatable iron pool of cultured hepatocytes from the increase of PG SK fluorescence after the addition of excess 2,2′-DPD. Using this method, we found 9.8 ± 2.9 μmol/L (mean ± SEM; n = 18) chelatable iron in rat hepatocytes, which constituted 1.0% ± 0.3% of the total iron content of the cells as determined by atomic absorption spectroscopy. The concentration of chelatable iron in hepatocytes was higher than the one in K562 cells (4.0 ± 1.3 μmol/L; mean ± SEM; n = 8), which were used for comparison. This method allowed us to record time courses of iron uptake and of iron chelation by different chelators (*e.g.*, deferoxamine, 1,10-phenanthroline) in single, intact cells. (HEPATOLOGY 1999;29: 1171-1179.)

The bulk of intracellular iron is safely bound to proteins and enzymes, but a very small amount of it belongs to a transition pool of chelatable iron that is thought to consist of iron associated with low-molecular-weight, low-affinity chelators such as citrate, phosphate, or adenosine triphosphate, and iron loosely bound to macromolecules such as proteins.[1,2] The cytosolic transition pool of iron is considered to constitute a link between transferrin-mediated iron uptake into cells on the one hand and intracellular storage proteins (*i.e.*, ferritin) and synthesis of iron-containing proteins (heme and non-heme iron proteins) on the other.[1-3] Iron constituting the transition pool is thought to prevail in the reduced form Fe(II) under physiological conditions.[2-7]

Iron ions in the transition pool are also capable of catalyzing the decomposition of H_2O_2 and organic hydroperoxides, yielding highly reactive oxygen species such as the hydroxyl radical.[1,3,8] These reactive species are able to initiate injurious processes like lipid peroxidation, oxidative alterations of proteins, DNA damage, and apoptosis.[1,9,10] Thus, they are considered to decisively contribute to a variety of liver diseases such as hepatitis, hemochromatosis, cirrhosis, and ischemia-reperfusion injury of the liver.[1-3,9,11] Recently, it has been suggested that in some of these pathological conditions, *e.g.*, nutrient deprivation, ethanol metabolism, oxidative stress, or ischemia, the key factor responsible for the increased formation of highly reactive oxygen species and cell injury is not an increased release of the superoxide anion radical and H_2O_2, but an increase in the intracellular amount of chelatable, "redox-active" iron.[12-16]

Until now, only a few methods of detecting the chelatable iron pool have been described,[12,15,17-21] but most of them remain insufficient in small amounts of biological material, and they are very prone to false readings arising from tissue processing (*e.g.*, proteolysis). In recent years, fluorescent dyes in combination with digital fluorescence microscopy have become increasingly important for the detection of intracellular ions. Recently, one method using the fluorescent dye, calcein, has been described for the assessment of the chelatable iron pool in cultured erythroleukemia K562 cells,[4] but this method did not prove to be well suited for similar measurements in hepatocytes. We therefore set about establishing a method of detecting chelatable iron in single, intact hepatocytes and also applied this method to human erythroleukemia K562 cells for comparison.

MATERIALS AND METHODS

Animals. Male Wistar rats (180-240 g) were obtained from the Zentrales Tierlaboratorium (Universitätsklinikum Essen). Animals were kept under standard conditions with free access to food and water. All animals received humane care in compliance with the institutional guidelines.

Abbreviations: MEM, minimum essential medium; 2,2′-DPD, 2,2′-dipyridyl; 4,4′-DPD, 4,4′-dipyridyl (dihydrochloride); DTPA, diethylenetriamine-pentaacetic acid; HBSS, Hanks' balanced salt solution; PG FL, phen green FL; PG SK, phen green SK; FL-DFO, fluorescein desferrioxamine; AAS, atomic absorption spectroscopy.

From the Institut für Physiologische Chemie, Universitätsklinikum, Essen, Germany.

Received August 3, 1998; accepted January 14, 1999.

Supported by the Deutsche Forschungsgemeinschaft (Klinische Forschergruppe Schock und Multiorganversagen; Schm 74/13-2).

Address reprint requests to: Dr. med. U. Rauen, Institut für Physiologische Chemie, Universitätsklinikum, Hufelandstr. 55, D-45122 Essen, Germany. E-mail: ursula.rauen@uni-essen.de; fax: 49-201-723 5943.

0270-9139/99/2904-0025$3.00/0

Chemicals. Leibovitz L-15 medium and α-minimum essential medium (α-MEM) were obtained from Gibco (Eggenstein, Germany). Collagenase (HEP plus), collagen (Type R), dexamethasone, and gentamycin were from Serva (Heidelberg, Germany), and dimethyl sulfoxide (DMSO), Triton X-100, nitric acid suprapur (65% vol/vol), and citric acid trisodium salt (dihydrate) were from Merck (Darmstadt, Germany). Deferoxamine mesylate (desferal) was purchased from Ciba-Geigy (Wehr, Germany). Fetal calf serum, 2,2′-dipyridyl (2,2′-DPD), 4,4′-dipyridyl (dihydrochloride) (4,4′-DPD), diethylenetriamine-pentaacetic acid (DTPA), ferrous ammonium sulfate, ferric chloride, 8-hydroxyquinoline, poly-L-lysine, and propidium iodide were obtained from Sigma (Deisenhofen, Germany), and 1,10-phenanthroline, 4,7-phenanthroline, and the ionophore, A23187, were from Sigma-Aldrich (Steinheim, Germany). 2,2′-DPD was dissolved in dimethyl sulfoxide, and DTPA was dissolved in NaOH (0.1 mol/L). 4,4′-DPD and desferal were dissolved in Hanks' balanced salt solution (HBSS), and 1,10-phenanthroline and 4,7-phenanthroline were dissolved in ethanol. Bovine serum albumin came from Behring Institute (Mannheim, Germany), and digitonin was from Fluka (Neu-Ulm, Germany). Falcon cell culture flasks and cell culture–grade Petri dishes (Falcon; 3004) were obtained from Becton Dickinson (Heidelberg, Germany), and glass coverslips were from Assistent (Sondheim/Röhn, Germany). Pluronic F-127, the ionophore, 4-bromo A-23187 (free acid), and the following fluorescent dyes for iron detection were purchased from Molecular Probes, Inc. (Eugene, OR): 1461-15-0 glycine,*N*,*N*′-((3′,6′-dihydroxy-3-oxospiro(isobenzofuran-1(3H),9′-(9H)xanthene)-2,7-diyl)bis(methylene))bis(*N*-carboxymethyl) (calcein, high purity) and its acetoxymethyl ester (calcein-AM), phen green FL (dipotassium salt) (PG FL) and its corresponding diacetate (PG FL, diacetate), phen green SK (dipotassium salt) (PG SK) and its diacetate (PG SK, diacetate), and fluorescein desferrioxamine (FL-DFO).

Cell Culture. Hepatocytes were isolated from male Wistar rats as described previously.[22] For the fluorescence measurements, 1.7×10^5 cells/cm^2 were seeded onto collagen-coated 6.15-cm^2 glass coverslips in Petri dishes; for the determination of the cellular iron content by atomic absorption spectroscopy (AAS), cell culture–grade Petri dishes (60-mm diameter) were used. Cells were cultured in L-15 medium supplemented with 5% fetal calf serum, L-glutamine (2 mmol/L), glucose (8.3 mmol/L), bovine serum albumin (0.1%), $NaHCO_3$ (14.3 mmol/L), gentamycin (50 mg/L), and dexamethasone (1 μmol/L) at 37°C in a 100% humidified atmosphere of 5% CO_2/95% air. Two hours after seeding, adherent cells ($0.24\text{-}0.57 \times 10^5$/cm^2) were washed three times with HBSS and supplied with fresh medium.

Human erythroleukemia K562 cells (DMS-No.: ACC 10) were obtained from the German cell culture collection (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, DSMZ, Braunschweig, Germany) and cultured as described by Breuer et al.[5]

Determination of Chelatable Intracellular Iron by Digital Fluorescence Microscopy. Experiments with hepatocytes were started 20 to 24 hours after isolation of the cells. The glass coverslips were transferred to a modified Pentz chamber and cells were washed twice with warm (37°C) HBSS (137 mmol/L NaCl, 5.4 mmol/L KCl, 1 mmol/L $CaCl_2$, 0.5 mmol/L $MgCl_2$, 0.4 mmol/L KH_2PO_4, 0.4 mmol/L $MgSO_4$, 0.3 mmol/L Na_2HPO_4, 20 mmol/L HEPES [pH 7.3]). The cells were loaded with FL-DFO (0.02-1 μmol/L) or with the membrane-permeable acetoxymethyl esters and diacetates, respectively, of PG SK (0.2-20 μmol/L), PG FL (0.2-2 μmol/L), or calcein (0.01-2 μmol/L) in HBSS at 37°C for 10 or 15 minutes. Cells were then washed three times with HBSS and incubated for another 15, 20, or 30 minutes in HBSS.

K562 cells (10×10^6 cells/mL, in suspension) were loaded with PG SK (20 μmol/L for 10 minutes) in HEPES-buffered (20 mmol/L, pH 7.3) α-MEM medium as used in Breuer et al.[4] After loading, cells were washed twice to remove PG SK by centrifugation (170*g* for 1 minute) and attached to poly-L-lysine–coated (5 μg/cm^2) glass coverslips 15 minutes before fluorescence measurements. Measurements were performed in 150 mmol/L NaCl, 10 mmol/L HEPES-Tris, pH 7.3 (HBS buffer).[5]

For PG SK fluorescence measurements (excitation maximum, 507 nm; emission maximum, 532 nm) and those of PG FL (excitation maximum, 492 nm; emission maximum, 517 nm), calcein (excitation maximum, 494 nm; emission maximum, 517 nm), and FL-DFO (excitation maximum, 493 nm; emission maximum, 515 nm; values as given by the manufacturer), an inverted microscope (Axiovert 135 TV; Zeiss, Oberkochen, Germany) equipped with the Attofluor imaging system (Atto Instruments, Rockville, MD) was used. Measurements were performed at 37°C using an excitation filter of 488 ± 10 nm, and monitoring the emission at 520 ± 20 nm using a bandpass filter.

The local distribution of PG SK in hepatocytes loaded with 20 μmol/L of the diacetate was determined by digitonin permeabilization (100 μmol/L) of the plasma membrane,[23] followed by lysis of the mitochondria with 0.2% Triton X-100.[24] A complete lysis of cells was obtained with 2% Triton X-100.[25] The detergents were added successively after the maximum of PG SK fluorescence in the cells had been obtained by addition of 5 mmol/L 2,2′-DPD.

The intracellular level of chelatable iron was manipulated 5 to 10 minutes after the beginning of the measurements by the addition of iron complexes and cell-permeable iron chelators to the supernatant. Fe(II) and Fe(III) stock solutions were always freshly prepared from ferrous ammonium sulfate and ferric chloride; ferrous ammonium sulfate was dissolved in distilled water together with citric acid trisodium salt dihydrate in equimolar concentrations, ferric chloride with the lipophilic iron ligand, 8-hydroxyquinoline, in dimethyl sulfoxide. In contrast to the usual incubation in HBSS, experiments in which Fe(III) was added were performed in HBSS that was substantially free from KH_2PO_4, $MgSO_4$, and Na_2HPO_4. These compounds were equimolarly replaced by KCl to maintain osmolarity.

The approximate cellular concentration of the fluorescent dye in the final procedure was determined as follows: hepatocytes were loaded with 20 μmol/L PG SK as described above. After the subsequent 15-minute incubation of the cells in HBSS, the supernatant was removed, the glass coverslips were washed, transferred into Petri dishes, and 1 mL of HBSS containing the iron chelator, 2,2′-DPD (5 mmol/L), and Triton X-100 (2%) was added (2,2′-DPD was included to avoid any quenching of the dye by contaminant iron in the buffer). The concentration of PG SK inside K562 cells was determined similarly using HBS buffer[5] instead of HBSS. The PG SK concentration in the cell lysate was determined from its fluorescence at room temperature compared with that of PG SK (free dye) standards in the same solution using a spectrophotofluorometer (Aminco-Bowman, model J4-8960E, SLM Instruments, Urbana, CA).

Determination of the Total Iron Content of Hepatocytes. The total iron content of hepatocytes was determined by atomic absorption spectroscopy (AAS) (Perkin Elmer, Überlingen, Germany, model 4100 ZL) using a modified standard method.[26,27] Cultured cells were washed three times with a HEPES-buffered solution (154 mmol/L NaCl, 10 mmol/L HEPES [pH 7.4]) prepared from distilled water that had been further purified by an 18 MΩ Milli-Q laboratory water purification system (Millipore, Eschborn, Germany); this is referred to below as 18 MΩ water. Cells were lysed overnight with 1.58 N HNO_3, and the iron content was determined by AAS. A cell-free sample was prepared the same way and used as a blank.

Cell Viability. The uptake of the vital dye, propidium iodide, was routinely determined at the end of the experimental procedures to detect loss of cell viability. Injurious effects of the fluorescent dye, PG SK (20 μmol/L), were further excluded by determination of released lactate dehydrogenase activity using a standard assay.

Fluorescence Measurements in a Cell-Free System. Spectra of free PG SK were scanned with a spectrophotometer (Perkin Elmer, type UV/VIS Lambda 40) and a spectrofluorometer (Firma Jobin Yvon, Longjumeau, France, type JY3) at a speed of 200 nm/min. Spectra were not corrected for light intensity or detector sensitivity.

In a cell-free system, we determined the effects of Fe(II) and Fe(III) and of Na^+ (NaCl), K^+ (KCl), Mg^{2+} ($MgCl_2$), Ca^{2+} ($CaCl_2$), Mn^{2+} ($MnCl_2$), Ni^{2+} ($Ni(SO_4)_2$), Cu^+ (CuCl), Cu^{2+} ($CuCl_2$), Co^{2+} ($CoCl_2$), and Zn^{2+} ($ZnCl_2$), the effect of the pH (pH 6.4-7.8), of the ionic strength (0 or 150 mmol/L K^+), and of the viscosity of the medium (altered by the addition of 1.75 mol/L sucrose)[28] on PG SK fluorescence. The measurements were performed at 37°C using two different media: 1) a simple buffered solution containing 2 mmol/L ascorbate (freshly prepared) in imidazole buffer (10 mmol/L; pH 7.2); and 2) a medium designed to simulate the composition of the cytosol: this "cytosolic" medium contained 100 mmol/L KCl, 5 mmol/L Na_2HPO_4, 2 mmol/L $MgCl_2$, the full amino acid composition contained in Eagle MEM (Sigma),[4] 6.85 mmol/L glucose, 1.5 mmol/L lactate, 230 μmol/L citrate, 138 μmol/L pyruvate, 2.99 mmol/L inorganic phosphate,[29] 4 mmol/L adenosine triphosphate,[30,31] 4.5 mmol/L glutathione,[32,33] and 2 mmol/L ascorbate[34,35] (both freshly prepared), 1.75 mol/L sucrose,[28] and 10 mmol/L imidazole buffer (pH 7.2).[36] All components of the media were dissolved in 18 MΩ water. The final media were treated with chelex[37] to minimize their heavy-metal contamination.

Aliquots (2 mL) of the media were transferred into the thermostated cuvette of the spectrofluorometer, and PG SK (final concentration, 0.5-80 μmol/L) was added and mixed with the medium. After the baseline fluorescence had been recorded for 5 minutes, the ions were added from their 100-fold stock solutions.

Statistics. All experiments with hepatocytes were performed in duplicate and repeated at least three times with cells from different animals; experiments with K562 cells were performed in duplicate and repeated at least three times, and experiments in a cell-free system were repeated at least twice. Traces shown in the figures are representative for all the corresponding experiments performed. The results are expressed as means ± SD or SEM.

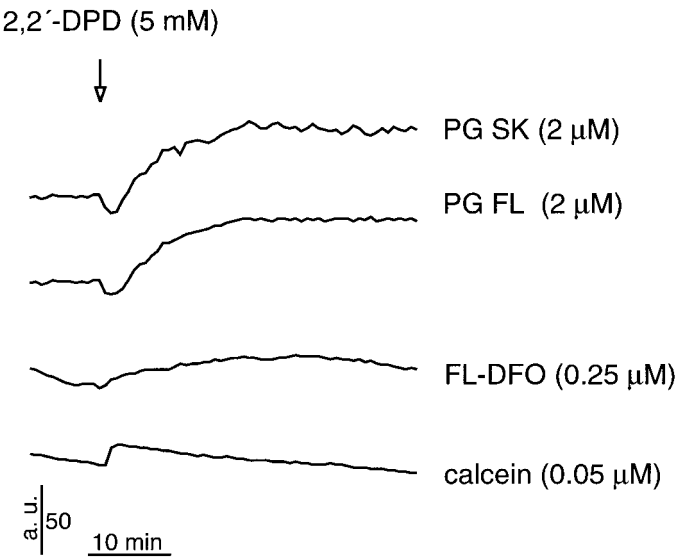

FIG. 1. Effect of the membrane-permeable transition metal chelator, 2,2′-DPD, on the intracellular fluorescence of cultured hepatocytes loaded either with PG SK (2 μmol/L), PG FL (2 μmol/L), FL-DFO (0.25 μmol/L), or calcein-AM (calcein, 0.05 μmol/L). The cells were loaded with the fluorescent indicators by incubation with the respective acetoxymethyl esters or diacetates for 10 minutes in HBSS (37°C). Cellular fluorescence was recorded at 60-second intervals using digital fluorescence microscopy (excitation, 488 ± 10 nm; emission, 520 ± 20 nm) and is given in arbitrary units (a.u.). 2,2′-DPD (5 mmol/L) was added as indicated by the *arrow*. Each trace shown is the average of 10 to 15 cells and is representative for at least 4 experiments using hepatocytes from different animals.

RESULTS

Evaluation of Different Fluorescent Probes for Determining Intracellular Chelatable Iron in Hepatocytes. To determine the chelatable iron pool of isolated rat hepatocytes, we tested the fluorescent probes, calcein, previously described as being suitable for the detection of iron in K562 cells,[4,5] and FL-DFO, PG SK, and PG FL, which have not yet been used for this purpose.

The addition of the membrane-permeable transition metal chelator, 2,2′-DPD (5 mmol/L), increased the intracellular fluorescence of all four dyes (Fig. 1, Table 1). This suggests that the fluorescence of the dyes was already partly quenched by iron chelation under control conditions and that redistribution of iron from the fluorescent indicator to a chelator like 2,2′-DPD abolishes this quenching, *i.e.*, "dequenches" the fluorescence. The increase in fluorescence upon the addition of 2,2′-DPD was far larger for PG SK– and PG FL–loaded hepatocytes than for FL-DFO– and calcein-loaded cells (Fig. 1 and Table 1). When cells were loaded with higher concentrations of PG SK, the relative change of PG SK fluorescence, and thus signal detectability, further increased, in contrast to calcein and FL-DFO, for which higher dye concentrations decreased signal detectability. Although there was almost no difference between PG SK and PG FL, PG SK was preferred, because PG FL appeared to require longer times to attain complete hydrolysis of the dye. Loading the cells for 10 minutes with 20 μmol/L of PG SK followed by an incubation of 15 minutes in dye-free HBSS gave optimum results (compare Figs. 1 and 2; Table 1). PG SK added at a concentration of 20 μmol/L had no cytotoxic effects on hepatocytes.

Dequenching of Cellular PG SK Fluorescence by Various Iron Chelators. While the addition of the membrane-permeable transition metal chelator, 2,2′-DPD, to PG SK–loaded hepatocytes led to a rapid increase in fluorescence, the nonchelating analogue, 4,4′-DPD, even provoked a slow decrease in fluorescence (Fig. 2). PG SK fluorescence also increased after addition of the transition metal chelator, 1,10-phenanthroline, or the clinically used Fe(III) chelator, deferoxamine, but was not affected by the nonchelating 1,10-phenanthroline analogue, 4,7-phenanthroline. The addition of 2,2′-DPD, 1,10-phenanthroline, or deferoxamine to unloaded hepatocytes, used as controls, had no effect on autofluorescence (data not shown).

While 2,2′-DPD and 1,10-phenanthroline quickly dequenched intracellular PG SK fluorescence, the dequenching occurred more slowly using the highly hydrophilic deferoxamine (Fig. 2). At lower concentrations of the chelators, dequenching occurred more slowly for all of them; however, it was again better for the lipophilic chelators (data not shown). Surprisingly, DTPA, which is considered to remain extracellularly, also caused a slow but significant increase in intracellular fluorescence.

The different chelators not only differed in the velocity with which the plateau was reached, but also in the maximum fluorescence intensity making the height of the plateau: deferoxamine and DTPA provided an incomplete dequenching in comparison with 2,2′-DPD and 1,10-phenanthroline, which had the largest effects of all chelators tested.

Quenching of Cellular PG SK Fluorescence by Increasing the Chelatable Iron Pool. Intracellular PG SK fluorescence was not only increased by iron chelators, but was also decreased by iron: the addition of either ferric chloride + 8-hydroxyquino-

TABLE 1. Intracellular Baseline Fluorescence Intensity, Relative Increase of Fluorescence Intensity Provided by 2,2′-DPD, and Leakage of the Dyes Used in Isolated Rat Hepatocytes

Dye	Concentration (μmol/L)	Amplification	Leakage (%/30 min)	Baseline Fluorescence Intensity (a.u.)	Maximal Fluorescence After 2,2′-DPD (a.u.)	Relative Increase (%)	
Calcein	0.05	99	20.8 ± 8.4	84.6 ± 21.0*	92.6 ± 23.8	8.7 ± 3.5	(n = 6)
FL-DFO	0.25	99	29.2 ± 6.5	96.2 ± 35.0*	99.4 ± 26.3	26.1 ± 7.5	(n = 3)
PG FL	2	99	1.1 ± 1.6	62.3 ± 10.5*	97.7 ± 19.2	53.6 ± 34.4	(n = 4)
PG SK	2	99	2.5 ± 3.8	66.8 ± 9.7*	109.0 ± 28.3	61.4 ± 25.2	(n = 5)
PG SK	20	90	8.6 ± 7.8	98.0 ± 21.8*	203.2 ± 27.3	115.5 ± 58.6	(n = 5)
No	—	99	—	40.7 ± 14.6	—	—	(n = 16)
No	—	90	—	21.7 ± 2.6	—	—	(n = 9)

NOTE. Cellular fluorescence of cultured hepatocytes loaded either with calcein-AM (calcein, 0.05 μmol/L), FL-DFO, 0.25 μmol/L, PG FL, 2 μmol/L, or PG SK, 2 or 20 μmol/L. The cells were loaded with the fluorescent indicators by incubation with the respective acetoxymethyl esters or diacetates for 10 minutes in HBSS (37°C). The cellular fluorescence was recorded using digital fluorescence microscopy (excitation, 488 ± 10 nm; emission, 520 ± 20 nm) and is given in arbitrary units (a.u.). It was necessary to adapt the amplification of the Attofluor imaging system of the microscope to the intracellular fluorescence intensity of hepatocytes loaded with 20 μmol/L PG SK. The leakage of the dyes (%/30 min), *i.e.*, combined leakage and photobleaching of the probes, was calculated from the decrease of fluorescence, *i.e.*, the baseline fluorescence intensity of loaded hepatocytes, before the transition metal chelator, 2,2′-DPD (5 mmol/L), was added to the incubation buffer of the cells. The increase of fluorescence intensity provided by the addition of 2,2′-DPD was obtained after complete equilibration corrected for leakage of the dye and is given as relative increase (%) of the baseline fluorescence. Values shown represent means ± SD of 3 to 16 experiments.

*Immediately before 2,2′-DPD was added.

line or of ferrous ammonium sulfate + citrate at concentrations of 16 μmol/L and 40 μmol/L, respectively, quenched the intracellular dye (Fig. 3). The progressive quenching eventually leveled off, suggesting that a steady state was reached. When the highly lipophilic complex of ferric chloride + 8-hydroxyquinoline was added to PG SK–loaded hepatocytes, a complete steady state of fluorescence intensity (Fig. 3A) was reached much faster than with the ferrous ammonium sulfate + citrate complex (Fig. 3B), suggesting differences in iron uptake. The response of PG SK fluorescence to the ferrous ammonium sulfate + citrate complex (10-40 μmol/L; data not shown) was only slightly dose-dependent, in contrast to the strongly dose-dependent response to the ferric chloride + 8-hydroxyquinoline complex (1-16 μmol/L) (Fig. 4). The extent of the quenching achieved with ferrous ammonium sulfate + citrate reached a maximum when ≥40 μmol/L Fe(II) was added (Fig. 3B), but was incomplete compared with the quenching after addition of 16 μmol/L ferric chloride + 8-hydroxyquinoline (Fig. 3A).

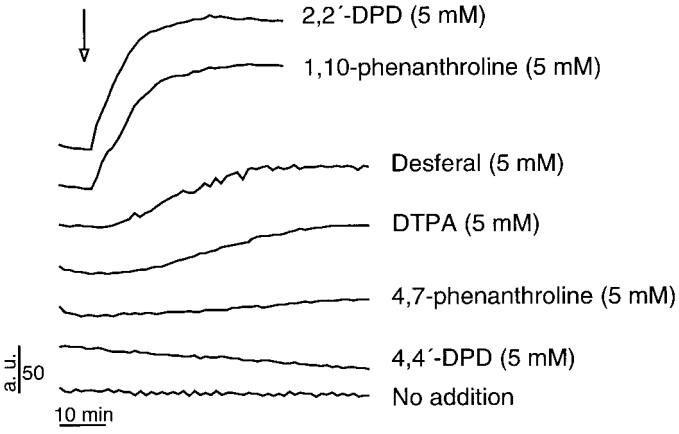

FIG. 2. "Dequenching" of the intracellular fluorescence of PG SK–loaded cultured hepatocytes by addition of various iron chelators. The membrane-permeable iron chelators, 2,2′-DPD (5 mmol/L), 1,10-phenanthroline (5 mmol/L), and deferoxamine mesylate (desferal, 5 mmol/L), the membrane-impermeable iron chelator, DTPA (5 mmol/L), and the nonchelating analogues to 2,2′-DPD and 1,10-phenanthroline, 4,4′-DPD (5 mmol/L) and 4,7-phenanthroline (5 mmol/L), were added (*arrow*) to hepatocytes loaded with PG SK by incubation with 20 μmol/L of the diacetate for 10 minutes in HBSS (37°C). Cellular fluorescence was recorded at 60-second intervals using digital fluorescence microscopy (excitation, 488 ± 10 nm; emission, 520 ± 20 nm) and is given in arbitrary units (a.u.). Each trace shown is the average of 10 to 15 cells and is representative for at least 4 experiments using hepatocytes from different animals.

Studies on Factors Influencing PG SK Fluorescence in a Cell-Free System. In addition to the experiments using intact cells, some properties of the dye were assessed in a cell-free system: free PG SK at a concentration of 50 μmol/L in a "cytosolic" medium (see Materials and Methods) had an excitation maximum at 526 nm and an emission maximum at 541 nm. Neither addition of 2,2′-DPD nor quenching by Fe(II) led to a shifting of the excitation or emission spectra. PG SK (50 μmol/L) was responsive to additions of Fe(II) as well as Fe(III), although Fe(II) was more effective (Table 2). The response to Fe(II) (0.5-100 μmol/L) was dose-dependent (see results below). Besides Fe(II) and Fe(III), PG SK fluorescence was markedly quenched by Cu(I), Cu(II), Co(II), and Ni(II), but was almost insensitive to all nonmetal ions tested (Table 2). In this "cytosolic" medium, far higher concentrations of iron were needed for quenching than in a simple buffered solution (data not shown)—most likely because organic iron chelators competed with the dye.

In the "cytosolic" medium, PG SK fluorescence and its quenching by Fe(II) was not affected by changes in pH (pH 6.4-7.8), but was slightly affected by changes in viscosity: when the viscosity of the medium was increased with 1.75 mol/L sucrose, PG SK fluorescence decreased by 4.5%. The ionic strength did not influence PG SK fluorescence and its quenching by Fe(II) as assessed by addition of KCl (150 mmol/L) in a simple buffered solution (see Materials and Methods).

Cellular Concentration and Compartmentalization of the Dye. When hepatocytes were loaded under the conditions described (20 μmol/L of the diacetate, 10 minutes, 37°C) we determined a mean intracellular PG SK content of 0.25 ± 0.02 nmol/10^6 cells, which corresponds to an intracellular concentration of 50 ± 3 μmol/L assuming a mean volume of 5 pl per hepatocyte.[38,39] Up to a magnification of 1,000×, there

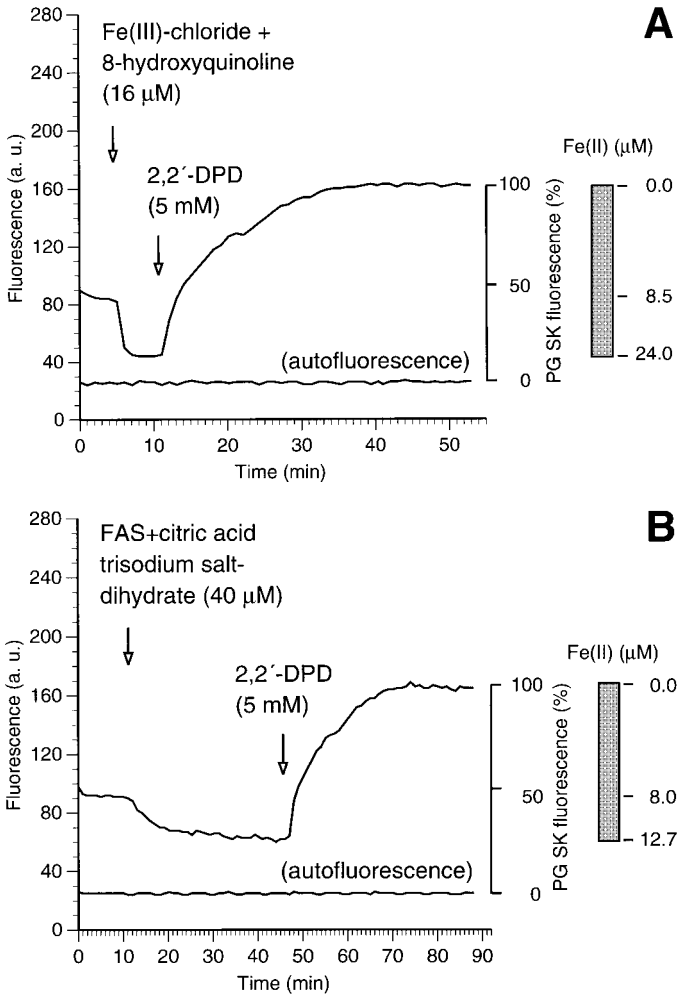

FIG. 3. Effect of an increase in the cellular chelatable iron on the fluorescence of intracellular PG SK in cultured rat hepatocytes. Hepatocytes were loaded with PG SK by incubation with 20 μmol/L of the diacetate for 10 minutes in HBSS (37°C). After establishing the baseline fluorescence, iron was added to the incubation buffer (HBSS) as (A) Fe(III) in a complex with 8-hydroxyquinoline, *i.e.*, in a form that is known to be membrane-permeable,[13,41,51] and (B) Fe(II) + citrate, which is known to be taken up by a transporter-mediated process, the non–transferrin-bound iron-uptake.[4,6,50] Afterward, the intracellular dye was "dequenched" by addition of excess 2,2′-DPD (5 mmol/L) to obtain the maximal signal (no quenching, *i.e.*, PG SK fluorescence intensity 100%). In addition, the autofluorescence from a parallel incubation of unloaded control hepatocytes is shown (PG SK fluorescence intensity 0%). Cellular fluorescence was recorded at 60-second intervals using digital fluorescence microscopy (excitation, 488 ± 10 nm; emission, 520 ± 20 nm) and is given in arbitrary units (a.u.). Each trace shown is the average of 10 to 15 cells and is representative for 5 experiments using hepatocytes from different animals. *Bars* give the approximated concentration of intracellular chelatable iron (μmol/L) as obtained using the calibration shown in Fig. 6.

were no detectable local accumulations of the dye, which appeared to be distributed homogenously inside hepatocytes before as well as after the addition of 2,2′-DPD (5 mmol/L) (Fig. 5). About 10% of the hepatocellular nuclei were nonfluorescent, whereas the rest of them were stained. After the addition of 100 μmol/L digitonin, 36.1% ± 4.7% of PG SK fluorescence disappeared within 2 to 3 minutes, a further 31.3% ± 0.1% of the fluorescence disappeared after addition of 0.2% Triton X-100, and 14.2% ± 3.3% of the fluorescence when 2% Triton X-100 was finally added; the remaining 18.4% ± 2.1% of the initial PG SK fluorescence appeared as diffuse background fluorescence (n = 3). These data suggest that ≈45% of the dye was located in the cytosol (comprising 57.9% ± 0.80% of hepatocellular volume[40]), ≈38% in the mitochondria (28.32% ± 0.50% of hepatocyte volume), and ≈17% in the remaining organelles (about 14% of hepatocellular volume).

Calibration Procedure. In situ calibration could not be performed in hepatocytes, because it requires a free equilibration of intra- and extracellular chelatable iron. Iron bound to 8-hydroxyquinoline is discussed as accumulating intracellularly,[41,42] thus ruling out the use of this complex in *in situ* calibration procedures. Furthermore, the ionophores, 4-bromo A-23187 and A-23187 (1-50 μmol/L), which have been described to permeabilize cells to Fe(II),[43,44] did not appear to equilibrate iron in hepatocytes when added to the supernatant. Therefore, we employed an *ex situ* calibration method.

We used free PG SK at a concentration of 50 μmol/L, *i.e.*, the concentration calculated to be present in PG SK–loaded hepatocytes (see above), and performed the calibration in the "cytosolic" medium at 37°C (Fig. 6). Using *ex situ* calibration, the chelatable iron pool of the cells was determined as follows: "unquenched," *i.e.*, maximum (100%) intracellular PG SK fluorescence was determined at the end of the experiment by addition of excess 2,2′-DPD (5 mmol/L). The autofluorescence from a parallel incubation of unloaded control hepatocytes was set at 0% PG SK fluorescence intensity. Increases and decreases of PG SK fluorescence in cells (%) were compared with the values obtained from the *ex situ* calibration (Fig. 6). We were able to estimate the

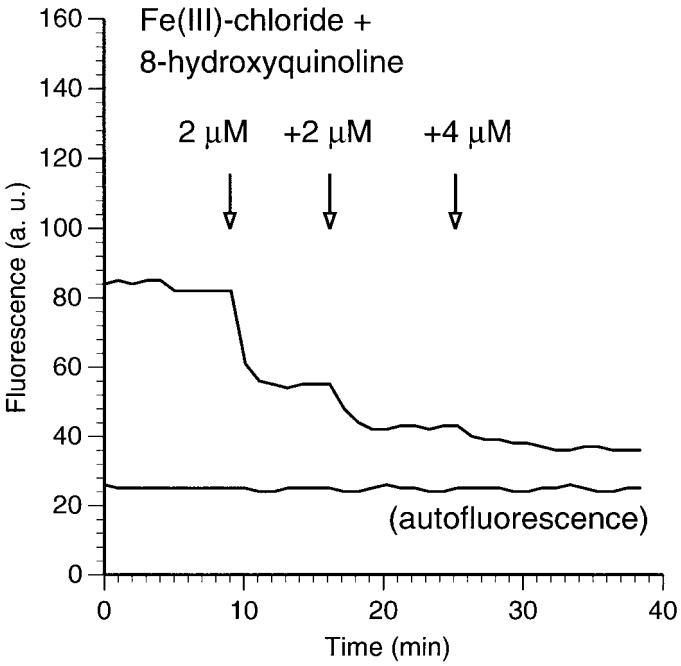

FIG. 4. Iron concentration–dependent quenching of PG SK fluorescence in cultured rat hepatocytes. The change in fluorescence intensity of PG SK–loaded (20 μmol/L) cultured hepatocytes after successive additions of Fe(III) complexed to the lipophilic iron chelator, 8-hydroxyquinoline, was monitored by digital fluorescence microscopy. In addition, the fluorescence from a parallel incubation of unloaded control hepatocytes is shown (autofluorescence; PG SK fluorescence intensity 0%). Cellular fluorescence was recorded at 60-second intervals (excitation, 488 ± 10 nm; emission, 520 ± 20 nm) and is given in arbitrary units (a.u.). Each trace shown is the average of 10 to 15 cells and is representative for 7 experiments using hepatocytes from different animals.

TABLE 2. Responsiveness of PG SK to Various Metal and Nonmetal Ions in a Cell-Free System

Ion	PG SK Fluorescence (% of baseline fluorescence) After Addition of 1 μmol/L	100 μmol/L
Ca(II)	99.4 ± 0.6	100.0 ± 1.2
Mn(II)	100.0 ± 0.02	100.0 ± 1.3
Fe(II)	92.0 ± 2.6	6.9 ± 1.3
Fe(III)*	95.5 ± 2.1	49.0 ± 6.4
Ni(II)	93.0 ± 0.8	70.8 ± 4.3
Cu(I)	97.0 ± 1.4	3.5 ± 0.3
Cu(II)*	97.6 ± 0.8	30.4 ± 0.6
Zn(II)	94.6 ± 2.3	95.7 ± 2.1
Co(II)	97.0 ± 2.1	71.6 ± 3.2

NOTE. Effect of various metal and nonmetal ions on PG SK fluorescence in a cell-free system recorded with a spectrofluorometer. PG SK (50 μmol/L) was dissolved in a chelex-treated medium (pH 7.2, 37°C) designed to simulate the composition of the cytosol (see Materials and Methods). The baseline fluorescence (excitation, 488 nm; emission, 520 nm) was recorded for 5 minutes, then the ions were added as their corresponding salts at the final concentrations indicated and rapidly mixed with the medium in the cuvette. The relative emission intensities shown are expressed in percent of the baseline fluorescence intensity (set at 100%) and were obtained after complete equilibration and corrected for photobleaching of the dye. Zero fluorescence is equal to fluorescence of medium without dye. Values shown represent means ± SD of 3 experiments.

*Measurements were performed in the absence of the reducing equivalents ascorbate and glutathione.

concentration of chelatable iron in hepatocytes (μmol/L) from the relative fluorescence intensity compared with the quenching in the *ex situ* calibration, *i.e.*, in the example shown in Fig. 3B, fluorescence 33 minutes after adding of Fe(II) corresponded to 25.4% of relative fluorescence intensity and thus to 12.7 μmol/L of chelatable iron.

Chelatable Iron Pool. Using the calibration procedure described above, we determined a concentration of chelatable iron of 9.8 ± 2.9 μmol/L (36.3% ± 10.8% relative fluorescence intensity; mean ± SEM; n = 18 experiments from 9 different animals) for isolated rat hepatocytes. The total iron content amounted to 4.7 ± 1.5 nmol/10^6 hepatocytes, as determined by atomic absorption spectrometry. Assuming a mean hepatocyte volume of 5 pl per cell,[38,39] these results suggest that the chelatable iron constitutes 1.0% ± 0.3% of total hepatocyte iron. Looking at the tracings of the individual hepatocytes, which showed cell-to-cell variation in the loading with PG SK (Fig. 5), the chelatable iron pool appeared to be similar in these cells, with concentrations distributed evenly around a mean of 10 μmol/L (range: 4.5-17.5 μmol/L; n = 141 cells from 9 different animals).

K562 Cells. This concentration of chelatable iron we found in cultured hepatocytes differs markedly from the value determined in erythroleukemia K562 cells (0.2-0.5 μmol/L) using the fluorescent indicator, calcein-AM.[5] To determine whether the two cell types differ with respect to their iron homeostasis or whether the two methods are measuring different pools, we extended our study to K562 cells. When K562 cells were loaded with PG SK (20 μmol/L), the baseline fluorescence was fairly high and dequenching with 2,2′-DPD (5 mmol/L) was weak (26.6%) in comparison with PG SK dequenching attainable in hepatocytes (Fig. 7; compare with Fig. 3). This suggests that PG SK fluorescence in K562 cells was quenched to a smaller extent under control conditions, *i.e.*, by a lower concentration of chelatable iron. In line with this result, the complex of ferric chloride + 8-hydroxyquinoline (10 μmol/L) quenched the fluorescence of PG SK–loaded K562 cells to a much greater extent than the fluorescence of PG SK–loaded hepatocytes (Fig. 7; compare with Fig. 3A). Quenching upon exposure to 10 μmol/L ferric chloride + 8-hydroxyquinoline was only partly (40%-50%) recovered with 2,2′-DPD (data not shown); this problem might be related to the cells, because it was also observed by Breuer et al. with calcein-loaded cells.[4] K562 cells, loaded with 20 μmol/L PG SK, contained 0.11 ± 0.01 nmol free dye per 10^6 cells, which corresponds to an intracellular concentration of 80 ± 10 μmol/L assuming a mean volume of 1.35 pl per cell.[45,46] Using an *ex situ* calibration with 80 μmol/L free PG SK, performed as described above (Fig. 6), we determined a concentration of 4.0 ± 1.3 μmol/L chelatable iron (73.4% ± 7.4% relative fluorescence intensity; mean ± SEM; n = 8) for K562 cells.

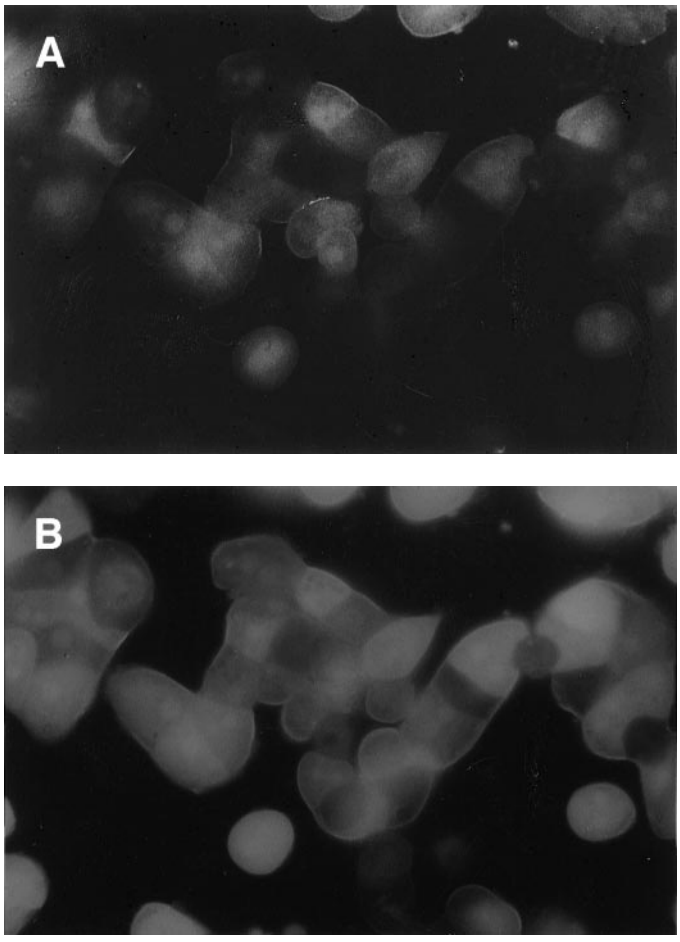

FIG. 5. Effect of the membrane-permeable transition metal chelator, 2,2′-DPD, on the fluorescence of cultured hepatocytes loaded with PG SK. Cells were loaded with PG SK by incubation with 20 μmol/L of the diacetate for 10 minutes in HBSS (37°C). Cellular fluorescence was excited at 488 ± 10 nm; for emission, a 520 ± 20-nm bandpass filter was used. The photographs (original magnification ×400) were taken (A) before addition of the transition metal chelator, 2,2′-DPD (5 mmol/L), *i.e.*, baseline fluorescence and (B) after complete equilibration following addition of 2,2′-DPD (5 mmol/L) (compare with Fig. 2).

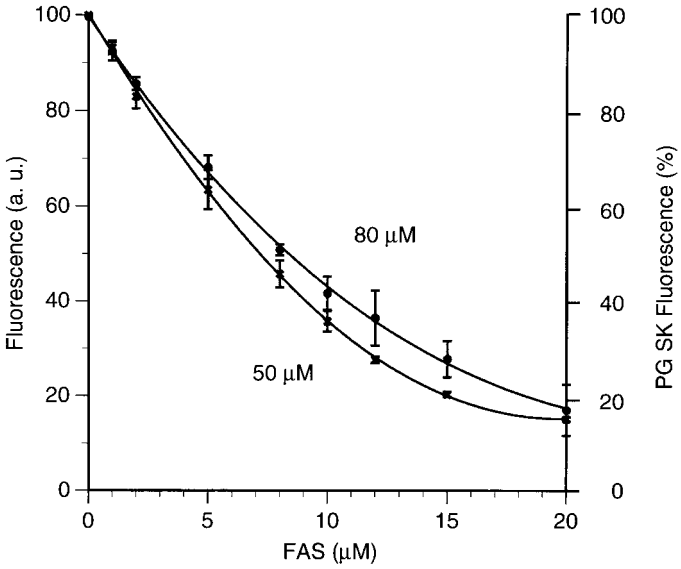

FIG. 6. *Ex situ* calibration of Fe(II)-induced quenching of PG SK fluorescence. In a cell-free system, free PG SK was dissolved at a final concentration of 50 μmol/L or 80 μmol/L in a chelex-treated medium (pH 7.2) designed to simulate the composition of the cytosol (see Materials and Methods). The iron content of this medium had been checked by AAS and was below 0.5 μmol/L. Fluorescence emission at 520 nm was immediately monitored using an Aminco-Bowman spectrofluorometer at 37°C. The baseline fluorescence was recorded for 5 minutes, then Fe(II) was added as ferrous ammonium sulfate (FAS) + citric acid trisodium salt dihydrate in increasing concentrations from freshly prepared 100-fold stock solutions and rapidly mixed with the medium in the cuvette. Values shown (in arbitrary units [a.u.]) were obtained after complete equilibration and corrected for photobleaching of the dye. Zero fluorescence is equal to fluorescence of medium without dye. Values shown represent means ± SD of 3 experiments.

DISCUSSION

In this study, we set up a method of detecting chelatable iron in intact hepatocytes by digital fluorescence microscopy using the fluorescent probe, PG SK. We determined a chelatable iron pool of 9.8 ± 2.9 μmol/L. It was possible to detect both increases and decreases in this pool at the single-cell level with high sensitivity.

Although in recent years, great effort has been devoted to developing suitable methods, the determination of the concentration of intracellular chelatable iron remained problematic: cell-disruptive methods such as homogenization or lysis followed by ultrafiltration and bleomycin-assay, high-performance liquid chromatography, electron paramagnetic resonance spectroscopy, affinity chromatography, or spectrophotometry of iron-chelator complexes require relatively large amounts of tissue and are prone to false readings. Because the chelatable iron pool is considered to amount to only 0.2% to 3.0% of the total cellular iron content,[12,14,18,47] a suitable method not only must be highly sensitive to chelatable iron, and to differentiate exactly between chelatable and tightly protein-bound iron ions, but it also must strictly avoid iron release from proteins into the chelatable pool. In tissue homogenates and cell lysates, a mobilization of iron from high-molecular-weight sources (*e.g.*, proteins like ferritin), *e.g.*, via proteolysis, is always a risk. These problems are reflected by the wide range of the values obtained in liver tissue and cultured hepatocytes using cell-disruptive methods (3.5-230 μmol/L).[12,14,17,19,20] The determination of the chelatable iron concentration in intact cells using ^{55}Fe or ^{59}Fe poses other problems. In contrast to fluorescence microscopy, iron adsorbed to extracellular surfaces contributes to the signal and therefore cannot be distinguished from the intracellular pool.[4] Furthermore, labeling often completely or partly fails to affect storage iron that interchanges with the chelatable iron pool.[2,3]

Because methods based on noninvasive fluorescence microscopy use living cells, disturbance from any pretreatment of biological samples (see above) is minimized. Changes in the chelatable iron pool and iron transport into cells can be recorded continuously (*i.e.*, on-line) with a high temporal resolution. Recently, a fluorimetric method using the fluorescent dye, calcein-AM, has been described for the assessment of the chelatable iron pool of human erythroleukemia K562 cells in suspension.[5] However, the method only produced signals slightly above background when applied to adherent hepatocytes (Fig. 1). The present method, in contrast, makes it possible to record changes to the chelatable iron pool in single, intact hepatocytes with high sensitivity.

PG SK fluorescence was strongly responsive to iron. Mobilization of iron loosely bound to ferritin by PG SK itself,

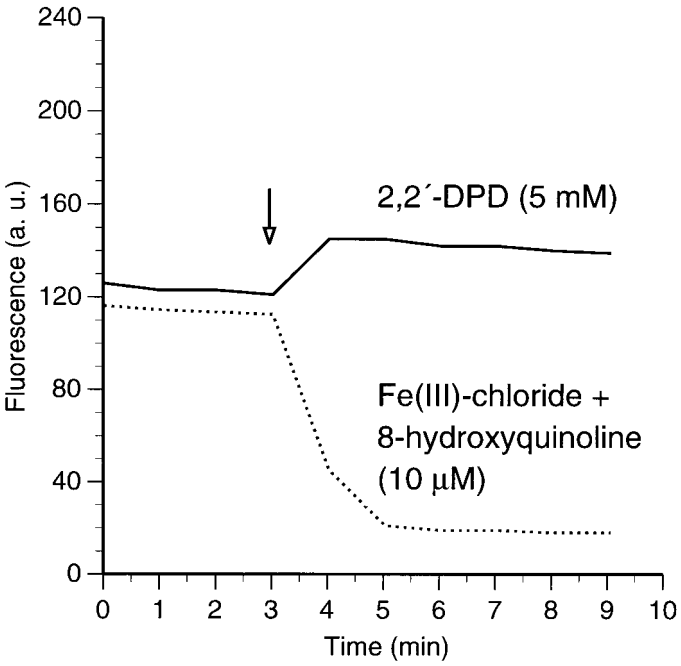

FIG. 7. Effect of a(n) decrease/increase in the cellular chelatable iron on the fluorescence of intracellular PG SK in human erythroleukemia K562 cells. K562 cells (in suspension) were loaded with PG SK by incubation with 20 μmol/L of the diacetate for 10 minutes in HEPES-buffered (20 mmol/L, pH 7.3) α-MEM medium (37°C). After loading, cells were washed twice to remove PG SK by centrifugation (170*g* for 1 minute) and attached to poly-L-lysine–coated (5-μg/cm^2) glass coverslips 15 minutes before fluorescence measurements. Measurements were performed in HBS buffer.[4] After establishing the baseline fluorescence, the intracellular dye was "dequenched" by addition of excess 2,2′-DPD (5 mmol/L) to obtain the maximal signal (no quenching, *i.e.*, PG SK fluorescence intensity 100%) or quenched by addition of ferric chloride + 8-hydroxyquinoline (10 μmol/L) to the incubation buffer (compare with Fig. 3A). Cellular fluorescence was recorded at 60-second intervals using digital fluorescence microscopy (excitation, 488 ± 10 nm; emission, 520 ± 20 nm) and is given in arbitrary units (a.u.). Each trace shown is the average of 3 to 15 cells and is representative for 8 experiments.

and therefore any overestimation of the chelatable iron pool, is unlikely, because the baseline fluorescence of PG SK–loaded hepatocytes remained almost stable. In the cell-free system, PG SK was also quenched by copper and (weakly) by Co^{2+} and Ni^{2+} (Table 2), but the contribution of these ions in the cellular system is likely to be negligible, because the intracellular concentrations of these metals are very low. Additionally, dequenching by deferoxamine in the cellular system suggests a major contribution by iron. Because an *in situ* calibration could not be performed as a result of the lack of complete transmembrane equilibration of Fe(II), we calibrated *ex situ*. In doing so, we took great care to simulate cytosolic conditions with regard to confounding factors. Because the concentration of chelatable Fe(III) is reported to be negligible relative to that of Fe(II),[2-7] we used Fe(II) only for calibration (Fig. 6).

Because actual cytosolic conditions can only be estimated, we regard the present method as very suitable for assessing relative changes to the chelatable iron pool in single cells or relative differences between different cell types/cell populations, but recommend that the limitations of *ex situ* calibration should be kept in mind when interpreting absolute values. We determined a total iron content of 4.7 ± 1.5 nmol/10^6 rat hepatocytes—which is in line with the literature[12,48,49]—suggesting that in these cells, 1.0% ± 0.3% of the total iron is chelatable, comparing well with the values given in the literature of 0.2% to 3.0%.[12,14,18,47]

The concentration of chelatable iron in cultured hepatocytes determined with our method (9.8 ± 2.9 μmol/L) differs markedly from the value determined in K562 cells (0.2-0.5 μmol/L) using the fluorescent indicator, calcein.[5] Differences between the two cell types account for part of this difference: using our method, we measured 4.0 ± 1.3 μmol/L chelatable iron in K562 cells, *i.e.*, a value that is significantly lower than in hepatocytes. However, part of the difference also appears to be inherent to the methods: the two methods might be measuring different pools, because the "chelatable iron" is a quantity that can at present only be defined operationally by the method used for its measurement. Thus, weak chelators like calcein might not detect iron ions complexed by organic chelators with a higher affinity,[4] while stronger chelators might remove iron from complexes that resist calcein. Furthermore, PG SK might also sample an iron pool that is not accessible to calcein as a result of other reasons: for example, while calcein has been described to be largely cytosolic,[4] PG appeared to be distributed evenly over cytosol and organelles. In addition, further differences might exist in the procedure used for calibration. Further studies and methods yet to be established are needed to resolve the question of what the differences between both methods are due to. The calcein and PG methods, likely to measure different pools, might even turn out to be useful as complementary methods in some cellular models.

The PG method allowed us to study the cell membrane permeability for iron complexes as well as the properties of various iron chelators by monitoring the time course of the change in PG SK fluorescence. It was suggested that the uptake of non–transferrin-bound iron into hepatocytes is mediated by a transmembrane carrier system (K_m = 16-19 μmol/L; V_{max} = 30-32 nmol · min^{-1} · g $liver^{-1}$).[6,50] This might explain why the Fe(II) + citrate complex only led to a slow quenching of intracellular PG SK fluorescence in comparison with the highly lipophilic Fe(III)–8-hydroxyquinoline complex, which is able to permeate the cell membrane.[13,41,51] Fe(III) added as a complex with 8-hydroxyquinoline is discussed as accumulating inside a mammalian epithelial cell line (CNCM I221) and inside murine L1210 lymphocytic leukemia cells,[41,42] and our results suggest that the same holds true for cultured rat hepatocytes and K562 cells (Figs. 3A and 4; compare with Figs. 6 and 7).

The addition of different iron chelators provided an increase in fluorescence that was different in time course and absolute amount (Fig. 2). These differences are most likely related to the chelators' membrane permeability, *i.e.*, lipid solubility, and secondary to their binding constants for Fe(II) and Fe(III) ions.[52] Deferoxamine is known to be highly hydrophilic, and therefore poorly membrane-permeable, which is reflected in the slow time course of "dequenching" PG SK fluorescence. Although deferoxamine is the stronger chelator (apparent binding constant K_{app} = 31.0), 2,2′-DPD (K_{app} = 17.3) and 1,10-phenanthroline (K_{app} = 21.0) complexed markedly more iron associated with PG SK than deferoxamine, suggesting that the latter have access to iron pools in hepatocytes that are not available for deferoxamine.

Highly hydrophilic iron chelators[52] like the impermeable[53] ferric chelator, DTPA (K_{app} = 27.0),[54] are reported to remain extracellular when added to cells, and DTPA has thus been used in cell cultures as a control displaying only extracellular effects.[4,55] DTPA affected the intracellular chelatable iron pool in our experiments (Fig. 2), and thus should be used with caution as an "extracellular" control. PG SK itself does not appear to inhibit iron-dependent processes like hydrogen peroxide toxicity (F. Petrat, unpublished results, October 1998).

Beside continuous measurement of iron uptake and testing the effectivity of iron chelators in cell culture models, the present method allows the study of heterogenous cell populations, and also the cellular and temporal correlation between changes in the chelatable iron pool and those in other parameters such as receptor status, or release of reactive oxygen species. The method should be suitable for various additional purposes like experimental simulations of pathological conditions that are discussed to increase the intracellular amount of chelatable iron. In addition, it might also be possible to determine the amount of chelatable iron within different intracellular compartments (like mitochondria or lysosomes) using the high spatial resolution of laser scanning microscopy.

Acknowledgment: The authors thank Mr. H. Diederichs (Institut für Arbeitsmedizin, Universitätsklinikum Essen) for performing the AAS measurements.

REFERENCES

1. Halliwell B, Gutteridge JMC. Role of free radicals and catalytic metal ions in human disease: an overview. Methods Enzymol 1990;186:1-85.
2. Young SP, Aisen P. The liver and iron. In: Arias IM, Boyler JL, Fausto N, eds. The Liver: Biology and Pathobiology. 3rd ed. New York: Raven, 1994:597-617.
3. Ryan TP, Aust SD. The role of iron in oxygen-mediated toxicities. Crit Rev Toxicol 1992;22:119-141.
4. Breuer W, Epsztejn S, Millgram P, Cabantchik IZ. Transport of iron and other transition metals into cells as revealed by a fluorescent probe. Am J Physiol 1995;268:C1354-C1361.
5. Breuer W, Epsztejn S, Cabantchik ZI. Iron acquired from transferrin by K562 cells is delivered into a cytoplasmic pool of chelatable iron(II). J Biol Chem 1995;270:24209-24215.
6. De Silva DM, Askwith CC, Kaplan J. Molecular mechanisms of iron uptake in eukaryotes. Physiol Rev 1996;76:31-47.

7. Meister A. Glutathione-ascorbic acid antioxidant system in animals. J Biol Chem 1994;269:9397-9400.
8. Stohs SJ, Bagchi D. Oxidative mechanisms in the toxicity of metal ions. Free Radic Biol Med 1995;18:321-336.
9. Bacon BR, Britton RS. The pathology of hepatic iron overload: a free radical-mediated process? HEPATOLOGY 1990;11:127-137.
10. Stadtman ER. Oxidation of free amino acids and amino acid residues in proteins by radiolysis and by metal-catalyzed reactions. Annu Rev Biochem 1993;62:797-821.
11. Bonkovsky HL, Banner BF, Rothman AL. Iron and chronic viral hepatitis. HEPATOLOGY 1997;25:759-768.
12. Baliga R, Ueda N, Shah SV. Increase in bleomycin-detectable iron in ischaemia/reperfusion injury to rat kidneys. Biochem J 1993;291:901-905.
13. Balla G, Vercellotti GM, Eaton JW, Jacob HS. Iron loading of endothelial cells augments oxidant damage. J Lab Clin Med 1990;116:546-554.
14. Öllinger K, Roberg K. Nutrient deprivation of cultured rat hepatocytes increases the desferrioxamine-available iron pool and augments the sensitivity to hydrogen peroxide. J Biol Chem 1997;272:23707-23711.
15. Sergent O, Morel I, Cogrel P, Chevanne M, Pasdeloup N, Brissot P, Lescoat G, et al. Increase in cellular pool of low-molecular-weight iron during ethanol metabolism in rat hepatocyte cultures. Biol Trace Element Res 1995;47:185-192.
16. Voogd A, Sluiter W, van Eijk HG, Koster JF. Low molecular weight iron and the oxygen paradoxon in isolated rat hearts. J Clin Invest 1992;90: 2050-2055.
17. Cairo G, Tacchini L, Pogliaghi G, Anzon E, Tomasi A, Bernelli-Zazzera A. Induction of ferritin synthesis by oxidative stress. Transcriptional and post-transcriptional regulation by expansion of the "free" iron pool. J Biol Chem 1995;270:700-703.
18. Gower JD, Healing G, Greene CJ. Determination of desferrioxamine-available iron in biological tissues by high-pressure liquid chromatography. Anal Biochem 1989;180:126-130.
19. Kozlov AV, Yegorov DY, Vladimirov YA, Azizova OA. Intracellular free iron in liver tissue and liver homogenate: studies with electron paramagnetic resonance on the formation of paramagnetic complexes with desferal and nitric oxide. Free Radic Biol Med 1992;13:9-16.
20. Nielsen P, Düllmann J, Wulfhekel U, Heinrich HC. Non-transferrin-bound-iron in serum and low-molecular-weight-iron in the liver of dietary iron-loaded rats. Int J Biochem 1993;25:223-232.
21. Rothman RJ, Serroni A, Farber JL. Cellular pool of transient ferric iron, chelatable by deferoxamine and distinct from ferritin, that is involved in oxidative cell injury. Mol Pharmacol 1992;42:703-710.
22. de Groot H, Brecht M. Reoxygenation injury in rat hepatocytes: mediation by O_2^-/H_2O_2 liberated by sources other than xanthine oxidase. Biol Chem Hoppe-Seyler 1991;372:35-41.
23. Pastorino JG, Snyder JW, Hoek JB, Farber JL. Ca^{2+} depletion prevents anoxic death of hepatocytes by inhibiting mitochondrial permeability transition. Am J Physiol 1995;268:C676-C685.
24. Davis MH, Altschuld RA, Jung DW, Brierley GP. Estimation of the intramitochondrial pCa and pH by fura-2 and 2,7 biscarboxyethyl-5 (6)-carboxyfluorescein (BCECF) fluorescence. Biochem Biophys Res Commun 1987;149:40-45.
25. Raju B, Murphy E, Levy LA, Hall RD, London RE. A fluorescent indicator for measuring cytosolic free magnesium. Am J Physiol 1989;256:C540-C548.
26. Brock TA, Brugnara C, Canessa M, Gimbrone MA. Bradykinin and vasopressin stimulate Na^+-K^+-Cl^- cotransport in cultured endothelial cells. Am J Physiol 1986;250:C888-C895.
27. Murphy E, Aiton JF, Horres CR, Lieberman M. Calcium elevation in cultured heart cells: its role in cell injury. Am J Physiol 1983;245:C316-C321.
28. Harootunian AT, Kao JPY, Eckert BK, Tsien RY. Fluorescence ratio imaging of cytosolic free Na^+ in individual fibroblasts and lymphocytes. J Biol Chem 1989;264:19458-19467.
29. Williamson DH, Brosnan JT. Metabolit-Gehalte tierischer Gewebe. In: Bergmeyer HU, ed. Methoden der enzymatischen Analyse. Vol 2, ed 3. Weinheim: Verlag Chemie, 1974;2317-2353.
30. Brecht M, Brecht C, de Groot H. Late steady increase in cytosolic Ca^{2+} preceding hypoxic injury in hepatocytes. Biochem J 1992;283:399-402.
31. Rauen U, Reuters I, Fuchs A, de Groot H. Oxygen-free radical–mediated injury to cultured rat hepatocytes during cold incubation in preservation solutions. HEPATOLOGY 1997;26:351-357.
32. Snel CAW, Pang KS, Mulder GJ. Glutathione conjugation of bromosulfophthalein in relation to hepatic glutathione content in the rat in vivo and in the perfused rat liver. HEPATOLOGY 1995;21:1387-1394.
33. García-Ruiz C, Morales A, Colell A, Ballesta A, Rodés J, Kaplowitz N, Fernández-Checa JC. Feeding *S*-adenosyl-L-methionine attenuates both ethanol-induced depletion of mitochondrial glutathione and mitochondrial dysfunction in periportal and perivenous rat hepatocytes. HEPATOLOGY 1995;21:207-214.
34. Forth W, Rummel W. Vitamine, Spurenelemente. In: Forth W, Henschler D, Rummel W, eds. Allgemeine und spezielle Pharmakologie und Toxikologie. 4th ed. Mannheim: Bibliographisches Institut, 1983:414-415.
35. Gries FA, Toeller M, Koschnisky T: Ernährungsstörungen. In: Siegenthaler W, Kaufmann W, Hornbostel H, Waller HD, eds. Lehrbuch der inneren Medizin. 6th ed. Stuttgart: Georg Thieme Verlag, 1984:282-284.
36. Lehninger AL, Nelson DL, Cox MM. Prinzipien der Biochemie. Heidelberg: Spektrum, 1994:255.
37. Evans PJ, Halliwell B. Measurement of iron and copper in biological systems: bleomycin and copper-phenanthroline assays. Methods Enzymol 1994;233:82-92.
38. Corasanti JG, Gleeson D, Boyer JL. Effects of osmotic stresses on isolated rat hepatocytes. I. Ionic mechanisms of cell volume regulation. Am J Physiol 1990;258:G290-G298.
39. Pfaller W, Willinger C, Stoll B, Hallbrucker C, Lang F, Häussinger D. Structural reaction pattern of hepatocytes following exposure to hypotonicity. J Cell Physiol 1993;154:248-253.
40. Blouin A, Bolender RP, Weibel ER. Distribution of organelles and membranes between hepatocytes and nonhepatocytes in the rat liver parenchyma. J Cell Biol 1977;72:441-455.
41. Jonas SK, Riley PA. The effects of the ligands on the uptake of iron by cells in culture. Cell Biochem Funct 1991;9:245-253.
42. Lin F, Girotti AW. Elevated ferritin production, iron containment, and oxidant resistance in hemin-treated leukemia cells. Arch Biochem Biophys 1997;346:131-141.
43. Epsztejn S, Kakhlon O, Glickstein H, Breuer W, Cabantchik ZI. Fluorescence analysis of the labile iron pool of mammalian cells. Anal Biochem 1997;248:31-40.
44. Nunez M-T, Cole ES, Glass J. The reticulocyte plasma membrane pathway of iron uptake as determined by the mechanism of a α,α'-dipyridyl inhibition. J Biol Chem 1983;258:1146-1151.
45. Dissing S, Hoffman R, Murnane MJ, Hoffman JF. Chloride transport properties of human leukemic cell lines K562 and HL60. Am J Physiol 1984;247:C53-C60.
46. Law F-Y, Steinfeld R, Knauf PA. K562 cell anion exchange differs markedly from that of mature red blood cells. Am J Physiol 1983;244:C68-C74.
47. Linder CM, Mulligan M, Henley D. Low molecular weight iron pools in tissues of the rat and pig. In: Urushizaki I, ed. Structure and Functions of Iron Storage and Transport Proteins. Amsterdam: Elsevier Science, 1983;465-471.
48. Adams PC, Reece AS, Powell LW, Halliday JW. Hepatic iron in the control of iron absorption in a rat liver transplantation model. Transplantation 1989;48:19-21.
49. Tyson CA, Green CE, Le Valley SE, Stephens RJ. Characterization of isolated Fe-loaded rat hepatocytes prepared by collagenase perfusion. In Vitro 1982;18:945-951.
50. Wright TL, Brissot P, Wai-Lan M, Weisiger RA. Characterization of non–transferrin-bound iron clearance by rat liver. J Biol Chem 1986;261: 10909-10914.
51. Oubidar M, Boquillon M, Marie C, Bouvier C, Beley A, Bralet J. Effect of intracellular iron loading on lipid peroxidation of brain slices. Free Radic Biol Med 1996;21:763-769.
52. Porter JB, Gyparaki M, Burke LC, Huehns ER, Sarpong P, Saez V, Hider RC. Iron mobilization from hepatocyte monolayer cultures by chelators: the importance of membrane permeability and the iron-binding constant. Blood 1988;72:1497-1503.
53. Schreiber B, Goldenberg H. Uptake of iron by isolated rat hepatocytes from a hydrophilic impermeable ferric chelate, Fe(III)-DTPA. Arch Biochem Biophys 1996;326:185-192.
54. Singh S, Hider RC. Colorimetric detection of the hydroxyl radical: Comparison of the hydroxyl-radical-generating ability of various iron complexes. Anal Biochem 1988;171:47-54.
55. Bottomley SS, Wolfe LC, Bridges KR. Iron metabolism in K562 erythroleukemic cells. J Biol Chem 1985;260:6811-6815.