Measurement of zinc in hepatocytes by using a fluorescent probe, Zinquin: relationship to metallothionein and intracellular zinc

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Zinquin [ethyl (2-methyl-8-*p*-toluenesulphonamido-6-quinolyloxy)acetate], a new intracellular zinc fluorophore, was used to reveal and to measure Zn in cultured rat hepatocytes before and after metallothionein (MT) induction. Hepatocytes labelled with an intense extranuclear fluorescence. Culture with combinations of Zn, dexamethasone and interleukin-6, increased intracellular MT by 24-fold, Zn 3-fold, and Zinquin fluorescence by approx. 2-fold above control values. Zinquin fluorescence correlated in descending order with the total cellular Zn ($r = 0.747$), exchangeable Zn ($r = 0.735$), soluble cytosolic Zn ($r = 0.669$) and MT ($r = 0.666$). When Zinquin was incubated with a cytosolic fraction of liver proteins before Sephadex G-75 column chromatography, it fluoresced with free, MT-incorporated and protein-bound Zn. Although only a slight attenuation of fluorescence was seen with high-molecular-mass protein-bound Zn, MT was degraded by 60% in the presence of Zinquin. The undegraded Zn-MT fluoresced at about 20% of the expected intensity. Although Zinquin fluoresces with all cytosolic Zn, caution is required when comparisons are made between samples with different concentrations of MT. This limitation was demonstrated by staining liver slices from adjuvant-treated rats where MT was increased 24-fold, intracellular Zn by 77%, but Zinquin fluorescence by only 19% above controls. Nevertheless, Zinquin should prove to be a useful tool for studying the distribution of Zn in living cells.

INTRODUCTION

Labile pools of intracellular Zn (*i*Zn) have been implicated in all phases of the cell cycle, including DNA synthesis and apoptosis, microtubule polymerization, gene expression, regulation of non-metalloenzymes, receptor down-regulation and neurotransmission (Elmes, 1977; Bettger and O'Dell, 1981; Assaf and Chung, 1984; Hesketh, 1985; Grummt et al., 1986; Chesters, 1989; Martin et al., 1991; Zalewski et al., 1991; Giannakis et al., 1991; Cousins and Lee-Ambrose, 1992).

Further investigation of the role of labile Zn in cell regulation would be facilitated by the introduction of specific *i*Zn fluorophores, analogous to the fluorescent indicators of intracellular free Ca^{2+} (Tsien et al., 1982). Toluenesulphonamidoquinoline [*N*-(6-methoxy-8-quinolyl)-*p*-toluenesulphonamide], a compound which fluoresces specifically on contact with Zn, has been used as a histochemical stain for Zn in sections of hippocampus and other tissues (Frederickson et al., 1987; Savage et al., 1987; Frederickson, 1989; Fliss et al., 1990). Because of its low affinity for Zn, relative to most metalloenzymes, it interacts mainly with loosely bound Zn in tissues (Frederickson, 1989). In order to facilitate the retention of this compound in living cells, the 6-methoxy group was changed to an ethyl ester (Zalewski et al., 1993). Cleavage of the ester by cytosolic esterases imparts a negative charge to the structure and impedes its efflux across the plasma membrane. We have used this new Zn-specific fluorophore, named Zinquin, to detect labile Zn in lymphoid and myeloid cells (Zalewski et al., 1993). Zinquin has the potential to define the relationship between the distribution of labile Zn and the major *i*Zn-binding protein, metallothionein (MT).

There is a major redistribution of Zn in the body via the plasma in certain physiological and pathological states, including stress, trauma, malignancy and inflammation. Under these conditions, the lowering of plasma Zn is thought to be caused by the induction of MT in the liver, which sequesters Zn from the plasma compartment (Cousins, 1985; Bremner and Beattie, 1990). In inflammatory disease, MT is regulated by a combination of affectors, including Zn, hormones and cytokines. We and others have shown that Zn, corticosterone (dexamethasone; Dex) and interleukin-6 (IL-6) act synergistically to induce MT synthesis in isolated and cultured rat hepatocytes (Schroeder and Cousins, 1990; Coyle et al., 1993a,b). Combinations of Zn + Dex + IL-6 induce MT concentrations 20-fold above controls. Newly acquired Zn is bound to liver MT, and this protein becomes the major pool of exchangeable Zn (Williams, 1984). By modulating the MT concentration in hepatocytes using various stimuli, it was possible to change *i*Zn concentration while maintaining a constant extracellular Zn concentration.

The aims of the study were: (1) to examine Zinquin labelling in hepatocyte cultures following MT induction *in vitro*; (2) to examine the relationship of Zinquin fluorescence to MT and *i*Zn pools; (3) to determine the interaction of Zinquin with hepatic Zn-binding proteins; (4) to investigate Zinquin labelling in liver slices from rats given an inflammogen to stimulate liver MT and increase *i*Zn.

MATERIALS AND METHODS

Materials used and their suppliers were: Williams medium E (Gibco), fetal-calf serum (Cytosystems Pty. Ltd., N.S.W., Australia), dexamethasone, fluorescamine and digitonin (Sigma), IL-6, fatty-acid-free BSA and collagenase H (Boehringer

Abbreviations used: AJ, adjuvant; Dex, dexamethasone; DMSO, dimethyl sulphoxide; IL-6, interleukin-6; *i*Zn, intracellular zinc; MT, metallothionein; HBSS, Hanks balanced salt solution.

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Mannheim, Castle Hill, N.S.W., Australia), ^{65}Zn as $ZnCl_2$ in 50 mM HCl (NEN Research Products, Biotechnology Systems Division, Du Pont, U.K.) and Zinquin [ethyl (2-methyl-8-*p*-toluenesulphonoamido-6-quinolyloxy)acetate] (synthesized at the Department of Chemistry, University of Adelaide). Zinquin was stored at 5 mM in dimethyl sulphoxide (DMSO) at −20 °C.

Male Dark Agouti rats weighing 250–400 g were supplied by the Institute of Medical and Veterinary Science Field Station, Gilles Plain, SA, Australia. Rats were housed on sawdust in plastic cages in an animal house maintained at 22 °C with a 14 h-light/10 h-dark cycle and had free access to water and a commercial non-purified diet (Milling Industries, Adelaide). This work was approved by the Animal Ethics Committee of the Institute of Medical and Veterinary Science, Adelaide.

Hepatocyte culture

Hepatocytes were isolated after liver perfusion with collagenase (Rofe et al., 1980). The liver cells were washed (three times) in Williams medium E containing Hepes buffer (20 mM), $NaHCO_3$ (10 mM) penicillin (240 mg/l) and gentamycin (160 mg/l) and resuspended in the same medium with the addition of 10 % (v/v) foetal-calf serum and insulin (25 units/l). Viability of isolated hepatocytes as determined by Trypan Blue exclusion was 80–95 %. Approx. 1×10^6 hepatocytes in 2 ml of medium were seeded on to collagen-coated flat-bottom culture wells (35 mm diameter; Corning, U.S.A.) and allowed to adhere to the surface at 37 °C in an atmosphere of 5 % CO_2 in air. In cultures used for Zinquin experiments, a collagen-coated glass coverslip (22 mm × 22 mm) was placed in the wells and used as a surface for cell adherence. After 4 h the medium was replaced with 2 ml of fresh medium without added insulin and the cells were cultured for 20 h. The culture medium was then replaced with 2 ml of Williams medium E containing Hepes (20 mM), $NaHCO_3$ (10 mM), penicillin (240 mg/l), gentamycin (160 mg/l) and fatty-acid-free BSA (0.5 %, w/v). Zn, hormones and IL-6 were then added. Zn added to the culture medium was bound to fatty-acid-free BSA as previously described (Coyle et al., 1993b). To some cultures was added 2 μCi of ^{65}Zn (40 mCi/mol of Zn). The basal Zn concentrations of the control medium with fatty-acid-free BSA (0.5 %, w/v) and that with the addition of 2 μCi of ^{65}Zn was < 1 μM and 25 μM respectively. Zn and Cu concentrations were determined by atomic-absorption spectroscopy. After incubation for a further 24 h, the cultures were treated as described below.

Rat thymocytes and splenocytes were prepared as described previously (Zalewski et al., 1993).

Measurements of *i*Zn

In studies measuring [*i*Zn], the medium was discarded and the hepatocytes were washed with 3 × 5 ml of PBS. Some monolayers were digested with 16 M HNO_3 (Aristar) and used in the measurement of total *i*Zn [*i*Zn (total)] and intracellular Cu. In other studies the cells were scraped from the plastic surface with a rubber policeman and lysed in 0.5 ml of Triton X-100 (0.25 %, v/v). The cytoplasmic soluble Zn [*i*Zn (lysate)] was determined in the supernatants of cell lysates that had been centrifuged at 13000 *g* for 5 min. The exchangeable Zn pool [*i*Zn (^{65}Zn)] was determined by measuring the ^{65}Zn present in both the cell lysate and culture medium with an LKB 1260 Multigamma counter.

MT was determined by a modified Cd/haemoglobin affinity assay (Eaton and Toal, 1982) on cell lysates that had been boiled for 2 min and then heat-labile protein had been removed by centrifugation at 10000 *g* for 1 min. MT concentration in hepatocyte cultures is reported as nmol of Cd bound/10^7 cells.

Preparation of liver slices from control and adjuvant-treated rats

Seven rats, weighing 190–350 g, were injected in the tail base with 100 μl of an arthritogenic adjuvant (AJ) composed of heat-killed *Myobacterium tuberculosis* H37 RA (Difco Laboratories, Detroit, MI, U.S.A.) (5 mg/ml) dispersed in squalene (Rofe et al., 1992). Five rats remained untreated and were used as controls. At 16 h after AJ treatment, the rats were anaesthetized with halothane and a blood sample was withdrawn by cardiac puncture and used in the measurement of serum Zn. The livers were excised, and three liver slices were prepared from each frozen section. A small portion of liver was stored at −20 °C for the measurement of MT and *i*Zn.

Fluorescent measurements with Zinquin

Frozen sections on microscope slides were overlaid with a solution of Zinquin (25 μM) in Hanks balanced salt solution (HBSS: NaCl, 137 mM; KCl, 5.4 mM; KH_2PO_4, 0.3 mM; Na_2HPO_4, 0.33 mM; $CaCl_2$,$2H_2O$, 1.23 mM; $MgSO_4$,$7H_2O$, 0.81 mM; D-glucose, 5.6 mM; $NaHCO_3$, 4.2 mM) for 40 min at 37 °C. Slides were washed with HBSS, mounted with a drop of Mounting Medium (Immunoconcepts) and coverslip, and fluorescence was examined with an Olympus microscope equipped with a UVB dichroic mirror and coupled to a videoPro Image Analysis Systems (Leading Edge Pty. Ltd., South Australia). Images of three fields were captured, and average fluorescence intensity of fields was determined within a rectangle of dimensions just smaller than the screen.

In cultures where cells were attached to glass coverslips, the medium was discarded and the cells were washed (three times) with HBSS or PBS. Coverslips were immersed in a solution of Zinquin (25 μM) in HBSS and incubated for 40 min at 37 °C. Coverslips were inverted on to microscope slides, and images of five fields per coverslip were captured. Average fluorescence intensity of individual cells was determined and background fluorescence substracted.

Spectrofluorimetry

Cells in suspension (5×10^6/ml in HBSS) were incubated with Zinquin (25 μM) in HBSS for 40 min at 37 °C. Cells were washed (three times) with HBSS, and fluorescence of 2 ml portions (5×10^6 cells/ml) in HBSS in cuvettes was measured at an excitation wavelength of 370 nm and emission wavelength of 490 nm (slit width 5–10 nm) in a Perkin–Elmer LS 50 luminescence spectrophotometer. $F_{max.}$ was determined by lysis of the cells with digitonin (50 μM) and saturation of the released Zinquin with excess 25 μM $ZnSO_4$. $F_{min.}$ was derived by the further addition of HCl (final concn. 100 mM) to these cell lysates to quench Zn-dependent fluorescence of the Zinquin.

Zinquin binding to protein in liver homogenates

Livers from Zn-treated rats were excised, rinsed in 150 mM KCl and homogenized in 3 vol. of 10 mM Tris buffer, pH 8.2. The homogenates were centrifuged at 20000 *g* for 30 min. The supernatants (1 ml) were incubated with 100 μl of 5 mM Zinquin or DMSO (control) for 30 min while shaking at 80 oscillations/min in a water bath at 37 °C. Molecular-mass profiles were estimated by separating the proteins by Sephadex G-75 gel filtration. A 0.5 ml sample was eluted with 40 mM phosphate

buffer, pH 7.4, from a 150 mm-length × 10 mm-diameter column, and 30 0.5 ml fractions were collected and analysed for MT, Zn, protein and Zinquin fluorescence. Total protein was determined by using fluorescamine (Udenfriend et al., 1972). Samples were diluted (1/50) in borate buffer (20 mM), pH 8.2, vortex-mixed with 2 vol. of fluorescamine [0.015 % (w/v) in acetone] and then read on a Perkin–Elmer spectrofluorimeter at excitation and emission wavelengths of 390 and 475 nm respectively. Human serum albumin was used as a standard. Zinquin fluorescence was determined at an excitation wavelength of 370 nm and emission wavelength of 490 nm.

Statistics

Results are reported as means ± S.E.M., and significant differences were assessed by Student's t test for independent means. Relationships between parameters were assessed by using Minitab software or Cricket Graph.

RESULTS

Fluorescence-labelling with Zinquin in hepatocytes

Rat hepatocytes incubated with 25 μM Zinquin had an intense extranuclear fluorescence, regardless of whether they were freshly prepared or cultured as monolayers (Figure 1). Some cells which stained less intensely appeared to be non-viable by their distinctive morphology, as seen during the Trypan Blue exclusion test.

Hepatocytes gave a more homogeneous and more intense fluorescence than other cells. The average fluorescence intensity of hepatocytes was 3.9- and 8.5-fold that of rat splenocytes and thymocytes respectively, as determined by image analysis.

Fluorescence of the cells was also quantified by spectrofluorimetry. As a preliminary experiment, we compared the basal fluorescence (F) of a suspension of hepatocytes with the maximal possible fluorescence ($F_{max.}$) after digitonin lysis and saturation of the cellular Zinquin with excess Zn and the minimum fluorescence ($F_{min.}$) after the subsequent addition of HCl to quench Zn-dependent Zinquin fluorescence (see the Materials and methods section). After subtraction of non-specific fluorescence of corresponding unloaded cells or lysates, we obtained values for F, $F_{max.}$ and $F_{min.}$ of 76.6 ± 5.6, 187.5 ± 14.3 and 1.5 ± 1.0 respectively. The relative sizes of F and $F_{max.}$ indicate that a substantial portion of the intracellular Zinquin was free of Zn. Since $F_{min.}$ was not significantly different from the fluorescence of the corresponding lysates from unloaded cells (due to autofluorescence and light-scattering of the cells and cuvettes), we can be confident that the free Zinquin is not contributing to F. The large excess of free Zinquin in the cell ensures that cellular uptake of Zinquin is not a limiting factor in the detection of *i*Zn.

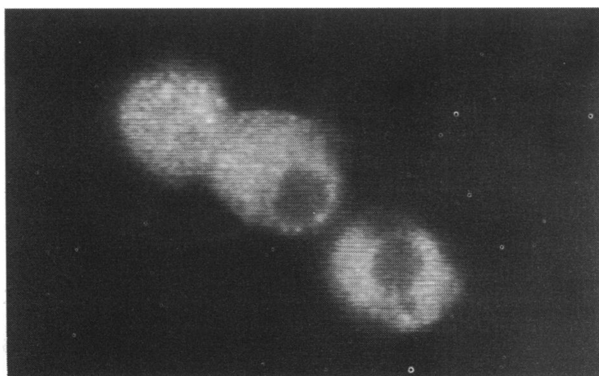

Figure 1 Zinquin fluorescence in cultured rat hepatocytes

Cells were incubated with 25 μM Zinquin under the conditions outlined in the Materials and methods section. Note the intense extranuclear fluorescence (magnification × 600).

Hepatocyte culture experiments

Zinquin fluorescence was investigated in cultured hepatocytes with various intracellular Zn and MT concentrations (Figure 2). In previous studies we have shown that combinations of Zn, Dex and IL-6 induce MT synthesis in cultured hepatocytes (Coyle et al., 1993a). Here, as in previous studies, the accumulation of MT with either Zn or Dex alone was small. Accumulations in the presence of Zn + Dex was 7.1- and 17.6-fold greater than the control (2.31 ± 0.22 nmol of Cd bound/10^7 cells) at Zn concentrations of 10 and 50 μM respectively. [Unless stated otherwise, the control (culture) contains no added Zn, dexamethasone or other effectors of MT synthesis.] IL-6 increased the MT response in the presence of Zn + Dex (50 μM Zn), with a maximum stimulation of 23.8-fold the control value. Total intracellular Zn [*i*Zn (total)] was increased above the control (29.3 ± 3.8 nmol/10^7 cells) in hepatocytes treated with 50 μM Zn alone, in combination with Dex or with Zn + Dex + IL-6 at 10–50 μM Zn. A maximum increase in *i*Zn of 2.8-fold the control value was obtained with Zn + Dex + IL-6 at the highest Zn concentration. Intracellular Cu concentration was 8.8 ± 0.7 nmol/10^7 cells ($n = 18$) and did not vary between treatment groups. Zinquin fluorescence was significantly raised in hepatocytes cultured with 50 μM Zn alone or in combination with Dex or Dex + IL-6, with a maximum 2.5-fold increase above the control (29.3 ± 3.8) with Zn + Dex + IL-6.

The relationship of Zinquin fluorescence to MT and *i*Zn was further examined. Other pools of *i*Zn were determined as follows: (1) Zn in supernatants of cell lysates was measured as an estimate of the soluble cytoplasmic *i*Zn pool [*i*Zn (lysate)]; (2) ^{65}Zn incorporation into hepatocytes was used as a measure of the exchangeable *i*Zn pool [*i*Zn (^{65}Zn)]. The comparison between *i*Zn, Zinquin fluorescence and MT was made on the combined data from the treatment groups previously described (Table 1). The *i*Zn (total), *i*Zn (lysate) and *i*Zn (^{65}Zn) correlated highly with each other. Zinquin fluorescence correlated highly in an exponential relationship with *i*Zn (total) and *i*Zn (^{65}Zn) but less strongly with *i*Zn (lysate) and with MT concentration, the latter being a logarithmic relationship. MT correlated highly in an exponential relationship with *i*Zn (lysate) and *i*Zn (^{65}Zn) and to a lesser extent with total *i*Zn.

Interaction of Zinquin with hepatocyte proteins *in vitro*

In order to determine the relative proportion of MT-associated Zn detected by Zinquin, the probe was incubated with a cytosolic fraction of liver proteins and then subjected to Sephadex G-75 column chromatography (Figure 3). The liver was obtained from a rat that had previously been injected intraperitoneally with $ZnSO_4$ in order to increase the MT concentration by more than 20-fold. The protein profiles for both the Zinquin and control (DMSO) were similar, with MT reaching a peak at fractions 16 and 15 respectively. Zinquin fluorescence was associated with high-molecular-mass protein fractions, MT and

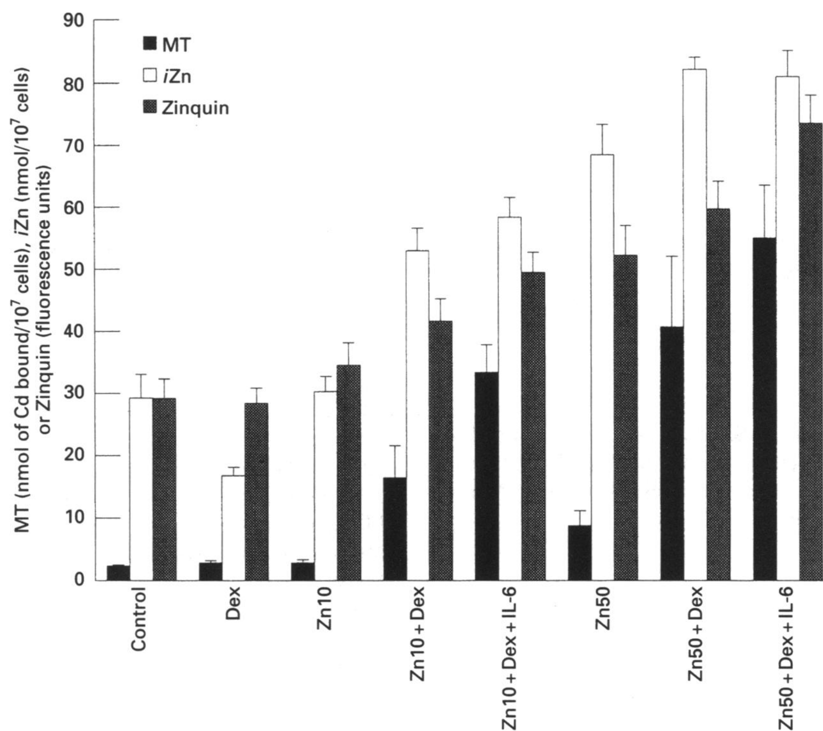

Figure 2 MT induction, iZn and Zinquin fluorescence in rat hepatocytes cultured for 24 h in the presence of no effector, Zn, Dex, Zn+Dex and Zn+Dex+IL-6

The concentration in cultures of the various inducers was serum 20% (v/v), Zn 10 or 50 μM, Dex 1 μM and IL-6 10^5 units/l. Further details are given in the Materials and methods section. The results are means ± S.E.M. of 4–10 independent culture experiments performed in triplicate.

Table 1 Relationship of Zinquin fluorescence to MT and Zn pools [iZn (total), iZn (lysate) and iZn (^{65}Zn)] in rat hepatocytes

Zinquin fluorescence, MT and cellular Zn values were derived from the combined data from the treatment groups previously described in the legend to Figure 2. Further details are given in the Materials and methods section. The regression equations chosen gave the highest regression coefficients: * significant regression slope at $P < 0.001$ (values in parentheses are numbers of independent observations).

Comparison	Regression equation	Regression coefficient (r)
iZn (total) versus iZn (lysate)	$y = 23.0 + 0,.89x$	0.820* (43)
iZn (total) versus iZn (^{65}Zn)	$y = 16.7 + 1.28x$	0.838* (42)
iZn (lysate) versus iZn (^{65}Zn)	$y = 1.35 + 1.20x$	0.771* (59)
Zinquin versus iZn (lysate)	$y = 26.9 \times 10^{0.0055x}$	0.669* (60)
Zinquin versus iZn (^{65}Zn)	$y = 22.5 \times 10^{0.0091x}$	0.735* (61)
Zinquin versus iZn (total)	$y = 20.7 \times 10^{0.0059x}$	0.747* (43)
Zinquin versus MT	$y = 25.7 + 20.2 \log x$	0.666* (68)
MT versus iZn (total)	$y = 1.24 \times 10^{0.0176x}$	0.681* (42)
MT versus iZn (lysate)	$y = 1.36 \times 10^{0.0236x}$	0.863* (59)
MT versus iZn (^{65}Zn)	$y = 1.04 \times 10^{0.0331x}$	0.771* (62)

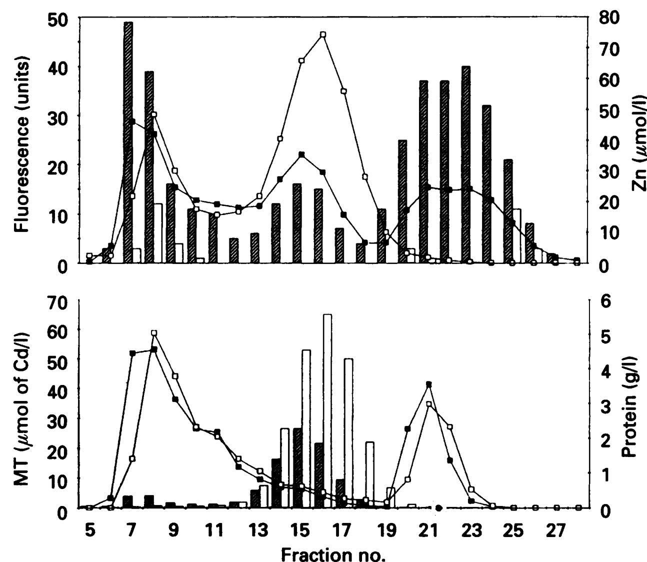

Figure 3 Typical Sephadex G-75 gel-filtration elution profile of hepatocyte homogenate incubated with Zinquin (▨, ■) and control (DMSO; ▭, □) under conditions described in the text

Upper panel: squares represent Zn concentrations; bars represent fluorescence. Note the increase in free zinc after incubation with Zinquin. Lower panel: squares and bars represent protein and MT concentrations respectively. Note the 60% decrease in MT in the presence of Zinquin.

lower-molecular-mass entities. Minimal background fluorescence was associated only with low- and high-molecular-mass fractions in the absence of Zinquin. In the controls some Zn was eluted in the high-molecular-mass region (fractions 7–12), but Zn was mainly found in the MT fractions (13–19). The presence of Zinquin, however, resulted in a 60 % decrease in the size of the MT peak, with a corresponding decrease in Zn concentrations.

This resulted in a large Zn peak in fractions 19–27, where only free ions or very small peptides are eluted. A minor MT peak at fractions 7–9 appears to represent MT polymerized by Zinquin treatment. Zinquin binds both free and protein-bound Zn with some quenching of fluorescence when Zn is bound to high-molecular-mass proteins and considerable quenching with Zn-MT. The findings that the MT peak was two-thirds smaller in the presence of Zinquin and was associated with a proportional increase in the free Zn fractions suggest that Zinquin binding removes Zn from MT, which is then degraded rapidly.

Image analysis of Zinquin-labelled liver slices

In order to determine whether the Zn status of the liver could be quantified with Zinquin, frozen sections of liver slices were prepared from control and AJ-treated rats and then stained with Zinquin. Fluorescence was quantified by Image Analysis. We have previously shown that, 16 h after administration of AJ to rats, liver MT increases by as much as 20-fold above control values (Rofe et al., 1992). A similar elevation of liver MT was observed 16 h after AJ in the present study (controls, 17 ± 4; AJ, 410 ± 40 nmol of Cd bound/g of liver). Plasma zinc concentrations were 18.6 ± 0.2 and 4.8 ± 0.7 μM in control and AJ-treated rats respectively, and hepatic [*i*Zn] was 417 ± 10 and 737 ± 45 nmol/g wet wt. of liver in controls and in treated rats respectively. There was a tendency towards increased fluorescence (19% higher) in liver slices from AJ-treated rats (40.8 ± 3.7) compared with controls (34.4 ± 3.9); however, this did not achieve significance.

DISCUSSION

These studies with Zinquin indicate that hepatocytes are rich in labile Zn, compared with lymphoid cells, and that Zn is distributed uniformly in the extranuclear region. The punctate fluorescence evident in lymphoid, myeloid cells and pancreatic islet cells was lacking in hepatocytes, and may indicate an absence of vesicular Zn in these cells. Lack of labelling in the hepatocyte nuclei is typical of other cell types that we have studied, and may indicate that the nucleus is depleted of Zn. This explanation, however, is contradicted by the observation that nuclei are very rich in exchangeable Zn, as shown by ^{65}Zn labelling studies (Cousins and Lee-Ambrose, 1992). Nuclear Zn has been estimated to constitute 25–40% of cellular Zn (Cousins, 1985). It is therefore more likely that, as a result of the cleavage of the ester group in Zinquin by cytosolic esterases, the compound becomes negatively charged and trapped within the cytosol, not being able either to exit the cell via the plasma membrane or to penetrate across the nuclear membrane.

Our estimation of *i*Zn in non-stimulated hepatocytes (29.3 ± 3.8 nmol/10^7 cells) is in agreement with that obtained by Schroeder and Cousins (1990) [27.2 ± 1.7 nmol/10^7 cells, assuming a protein content of 17 mg/10^7 cells (Berry et al., 1991)]. This *i*Zn concentration is approx. 30-fold greater than that found in human leucocytes (1.0 nmol/10^7 cells; Goode et al., 1989). In agreement with this, hepatocytes fluoresced intensely with Zinquin, compared with lymphocytes. Why hepatocytes are rich in Zn compared with lymphoid cells is not clear, but this probably reflects the large range of metabolic processes carried out by the liver. Basal MT concentrations may also be higher in hepatocytes relative to lymphoid cells, although in the present study Zn bound to MT in unstimulated hepatocytes only accounted for less than 1% of the total *i*Zn pool.

Hepatocytes differ from most cells in that their Zn content is markedly influenced by the synthesis of MT, which is induced by a combination of inflammatory stimuli. The rise in liver MT results in sequestration of Zn from the plasma compartment into which flows labile Zn from other tissues, resulting in redistribution of body Zn. Several hypotheses have been advanced on the importance of Zn redistribution in inflammatory disease. Increased liver Zn may be necessary to activate some metalloenzymes. In this regard, Zn-MT has been found to re-activate various Zn-dependent enzymes *in vitro* (Udom and Brady, 1980; Li et al., 1980; Winge and Miklossy, 1982). Zn may also be required for the increased protein synthesis in the liver during the acute-phase response (Solomons, 1988). Zn is a pivotal regulator of cell growth and death, being a cofactor in mitosis of hepatocytes and other cells (Chesters, 1989; Kobusch and Bock, 1990), as well as suppressing the endonucleases that mediate DNA fragmentation in apoptotic cell death (Giannakis et al., 1991; Zalewski et al. 1991). The homoeostatic balance between cell generation and death in the liver may therefore be influenced by the influx of zinc.

The relationship of Zinquin labelling to MT concentration and *i*Zn in hepatocytes treated with various stimuli was examined. It has previously been shown that combinations of Zn, Dex and IL-6 act in synergy to induce MT synthesis in isolated and cultured rat hepatocytes (Schroeder and Cousins, 1990; Coyle et al., 1993a,b). Combinations of Zn, Dex and IL-6 were used in the present study (Figure 2), and it was found that Zinquin fluorescence correlated more strongly with total *i*Zn than with MT. The disparity between Zinquin labelling and MT concentration was most marked in treatment groups with single effectors (control, Dex or Zn) compared with those with combinations (Zn + Dex, Zn + Dex + IL-6). For example, 2–3-fold increases in Zinquin labelling and in *i*Zn were obtained whether MT concentrations were 4-fold (50 μM Zn) or 24-fold (Zn50 + Dex + IL-6) the control value. These differences highlight the poor induction of MT obtained with Zn alone, compared with combinations of effectors which act in synergy on the MT gene. The raised *i*Zn obtained in cells incubated in the presence of 50 μM Zn is likely to be due to the mass action of the higher Zn concentration. The correlation between Zinquin and MT may be affected by several factors, including the high basal level of *i*Zn that appears to be independent of MT concentration, the variability of both the Zinquin and MT assays, and the difference in the induction of MT at similar *i*Zn concentrations. The 2–3-fold increase in *i*Zn concentration with Zn + Dex + IL-6 can be accounted for by the Zn bound to MT. Here, MT-bound Zn represented 102–107% of the change in the total *i*Zn pool elicited with Zn + Dex + IL-6 at 10 and 50 μM Zn respectively. Intracellular Cu concentration was only one-third of the *i*Zn concentration in the controls, and did not vary between treatment groups, excluding any effect of Cu on MT lability or measurement. These findings reflect the strong relationship between hepatic Zn uptake and metallothionein synthesis that are observed *in vivo*.

Further examination of the relationship between Zinquin labelling, MT and *i*Zn was made by combining the results for all treatment groups (Table 1). Zinquin labelling and MT were compared with three estimations of *i*Zn: the total pool, the cytosolic pool and an exchangeable Zn pool. All three *i*Zn pools were highly related to each other and with Zinquin labelling. The highest correlation was between Zinquin labelling and the total *i*Zn pool, indicating that the probe reacts with Zn bound to nearly all forms of intracellular ligands rather than to freely exchangeable cytosolic Zn only. This finding is supported by studies where hepatocyte proteins were incubated with Zinquin

before Sephadex G-75 gel filtration (Figure 3). Zinquin fluorescence was found over the broad spectrum of molecular masses. Interestingly, Zinquin removed a large proportion of Zn from MT, resulting in the appearance of free Zn bound to Zinquin, and a corresponding decrease in the MT concentration, probably as a result of the degradation of the more labile apo-MT (Nath et al., 1988). There appeared to be no removal of Zn from higher-molecular-mass proteins, however. It has been suggested that MT-bound Zn is part of the labile pool in cells (Williams, 1984). Our indirect evidence from gel-filtration studies, that Zinquin successfully competes with MT for its bound Zn, supports this concept. Zn associated with MT may be en route to incorporation in other proteins and cellular organelles. Zinquin will enable us to investigate these issues further.

Image analysis of Zinquin-labelled liver slices did not quantitatively reflect the tissue Zn concentration. The fluorescence observed in samples prepared from AJ-treated rats was similar to those from control rats, although the zinc concentrations in the livers from AJ-treated animals were almost double that in controls. Our inability to quantify Zn status in tissue slices with Zinquin may result from variable quenching of fluorescence by Zn-binding ligands in both the cell and matrix (Zn-MT, which represented 58% of the total hepatic Zn in AJ-treated rats, compared with 6% in controls, was found to cause greater quenching than other Zn-binding ligands; see studies *in vitro*, Figure 3) and/or the non-homogeneity of the sample and Zinquin staining in frozen sections.

Despite obvious limitations in quantification, Zinquin labelling is a unique tool providing high-resolution images of all Zn distributed within tissues and cells.

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