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Demonstration of nuclear compartmentalization of glutathione in hepatocytes

GIORGIO BELLOMO*, MARIAPIA VAIRETTI†, LUCIANNA STIVALA‡, FRANCESCA MIRABELLI*, PLINIO RICHELMI†, AND STEN ORRENIUS§¶

*Dipartimento di Medicina Interna e Terapia Medica, Clinica Medica I, †Istituto di Farmacologia Medica II, and ‡Istituto di Patologia Generale, University of Pavia, I-27100, Pavia, Italy; and §Department of Toxicology, Karolinska Institutet, S-104 01, Stockholm, Sweden

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ABSTRACT **The intracellular distribution of glutathione (GSH) in cultured hepatocytes has been investigated by using the compound monochlorobimane (BmCl), which interacts specifically with GSH to form a highly fluorescent adduct. Image analysis of BmCl-labeled hepatocytes predominantly localized the fluorescence in the nucleus; the nuclear/cytoplasmic concentration gradient was approximately three. This concentration gradient was collapsed by treatment of the cells with ATP-depleting agents. The uneven distribution of BmCl fluorescence was not attributable to (*i*) nonspecific interaction of BmCl with protein sulfhydryl groups, (*ii*) any selective nuclear localization of the GSH transferase(s) catalyzing formation of the GSH–BmCl conjugate, or (*iii*) any apparent alterations in cell morphology from culture conditions, suggesting that this distribution did, indeed, reflect a nuclear compartmentalization of GSH. That the nuclear pool of GSH was found more resistant to depletion by several agents than the cytoplasmic pool supports the assumption that GSH is essential in protecting DNA and other nuclear structures from chemical injury.**

The tripeptide glutathione (GSH) is involved in many important cellular functions, including maintenance of the thiol-disulfide status of structural and soluble proteins, detoxication of electrophiles, and reduction of hydroperoxides (1–4). The intracellular concentration of GSH in most mammalian cells is relatively high (millimolar range) and is kept at these levels by continuous synthesis (5).

Approximately 10–20% of total cellular GSH in rat liver is sequestered in the mitochondrial matrix (6, 7). The size of this pool depends on cytosolic GSH synthesis (8) and the active transport of GSH into mitochondria via a multicomponent system recently described (9).

Conventional cell-fractionation studies have not provided evidence for the existence of functionally distinct pools of GSH in hepatocytes other than those in the cytosol and mitochondria. Despite the known functions of GSH in DNA synthesis (10) and protection from oxidative DNA damage (11), little is known about the nuclear localization of GSH and the factors regulating the nuclear GSH level. Tirmenstein and Reed (12), using fractionation and centrifugation techniques in nonaqueous medium, measured the nuclear GSH content in rat kidney and found values similar to those in the cytosol. Other fractionation techniques (such as selective permeabilization of cell constituents with various detergents) provided equivocal results (13, 14). However, the latter investigations have suggested that a nuclear pool of GSH may exist in intact cells.

Recent advances in image-analysis technology, together with the development of additional, nontoxic fluorescent indicators that can be used in intact cells, have facilitated an enormous input into the study of various aspects of cell physiology (15, 16). Among the available indicators are monobromo- and monochloro-derivatives of bimanes, which are essentially nonfluorescent by themselves but which develop a strong blue fluorescence upon binding to thiol groups (17, 18). Interestingly, the rate of reaction of monochlorobimane BmCl with GSH and other thiols is relatively low, whereas the rate of formation of the GSH adduct is increased markedly in the presence of GSH transferase(s) (19). This property has made it possible to use BmCl to fluorescently label GSH in living cells.

In the present study, we have used image-analysis techniques to investigate the intracellular distribution of GSH in cultured hepatocytes with BmCl as a fluorescent indicator. Our findings indicate that GSH is concentrated in the nucleus and that the nuclear/cytoplasmic GSH concentration gradient is maintained by active mechanisms.

MATERIALS AND METHODS

Materials. Collagenase H was obtained from Boehringer Mannheim; BmCl, monobromobimane (BmBr), fluorescein isothiocyanate (FITC), and Hoechst 33258 were purchased from Molecular Probes. Cell culture media, antibiotics, and fetal bovine serum were obtained from Flow Laboratories. All other reagents were of the highest grade of purity available and were obtained from local suppliers.

Hepatocyte Isolation and Culture. Hepatocytes were isolated from male Wistar rats (200–250 g, fed ad libitum) by collagenase perfusion of the liver (20) and incubated at 5×10^6 cells per ml. In cell culture experiments, hepatocytes were plated on collagen-coated glass coverslips in 60 × 10 mm Petri dishes at 50,000 cells per ml in Williams E medium (Sigma)/10% fetal bovine serum/antibiotics. Cells were cultured in 5% CO_2/95% air for 24 hr unless otherwise indicated.

Preparation of Subcellular Fractions. After perfusion for 3 min with cold 0.9% NaCl to remove blood, livers were excised, minced, and homogenized in cold KTM buffer (115 mM KCl/10 mM Tris, pH 7.2/3 mM $MgCl_2$). The procedure of Spelberg *et al.* (21) was used to isolate nuclei; other subcellular fractions were isolated by conventional fractionation techniques. After preparation, the cytosolic fraction was dialyzed overnight against 1000 vol of KTM medium to remove GSH and other low-molecular-weight thiols.

Fluorescence Measurements in Individual Hepatocytes. The equipment used for single-cell measurement of fluorescence consisted of a Spex Fluorolog-2 spectrofluorometer (Spex Industries, Edison, NJ) connected to a Nikon Diaphot inverted microscope. Fluorescent images were obtained using an MTI SIT66 camera and processed by an ITI 150 image-processing system (Spex Industries). Cells grown on cover-

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Abbreviations: GSH, glutathione; BmCl, monochlorobimane; BmBr, monobromobimane; DEM, diethyl maleate; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; FITC, fluorescein isothiocyanate.

¶To whom reprint requests should be addressed.

slips were rinsed twice in Krebs–Henseleit buffer (20) and then mounted in a thermostated flow-through chamber and placed on the microscope stage. For bimane fluorescence, cells were incubated for 90 sec with either 80 μM BmCl or 200 μM BmBr and then washed in Krebs–Henseleit buffer. Cells were excited at 395 nm, and the fluorescence was imaged through a 445-nm dichroic mirror and a 470-nm barrier filter. For Hoechst 33258 fluorescence, cells were incubated for 120 sec with Hoechst 33258 at 2 μg/ml and rinsed twice. The excitation wavelength was 352 nm, and images were acquired by using a 410-nm dichroic mirror and a 420-nm long-pass emission filter. For FITC fluorescence, cells were incubated for 120 sec with 50 μM FITC and rinsed twice. The excitation wavelength was 490 nm, and images were obtained by using a 500-nm dichroic mirror and a 520- to 560-nm emission filter. Thirty-two to 64 frames were averaged for each acquisition cycle, and the acquired image was then processed; fluorescence intensity was measured according to a pseudocolor scale.

Other Assays. Bimane fluorescence in all suspensions or subcellular fractions was measured by using a Perkin–Elmer LS-5B fluorometer operating at 385-nm (excitation) and 470-nm (emission) wavelengths. Specific information for each set of experiments is reported in the table and figure legends. Intracellular GSH content was measured as described in ref. 22. Protein concentration was assayed according to Lowry *et al.* (23).

RESULTS

The specificity of the fluorometric method used to investigate GSH distribution in hepatocytes is illustrated in Fig. 1. Sequential additions of GSH to an incubation buffer containing BmCl did not appreciably modify the baseline fluorescence (Fig. 1*A*). However, the inclusion of small amounts of liver cytosol rapidly increased fluorescence. In contrast, BmBr reacted nonenzymatically with both GSH and sulfhydryl groups in cytosolic proteins, as revealed by the fluorescence increase seen directly upon addition of GSH and/or cytosol to an incubation buffer containing BmBr (Fig. 1*B*).

To investigate the distribution of GSH in hepatocytes, cells grown on coverslips for 24 hr were incubated with 80 μM BmCl at room temperature for 90 sec before fluorescence examination and image analysis. Control experiments showed that formation of the GSH–BmCl conjugate was maximal at 40 μM extracellular BmCl ($K_m \cong 10$ μM) and was completed in <60 sec. As illustrated in Fig. 2, image analysis of BmCl-labeled hepatocytes revealed an uneven distribution

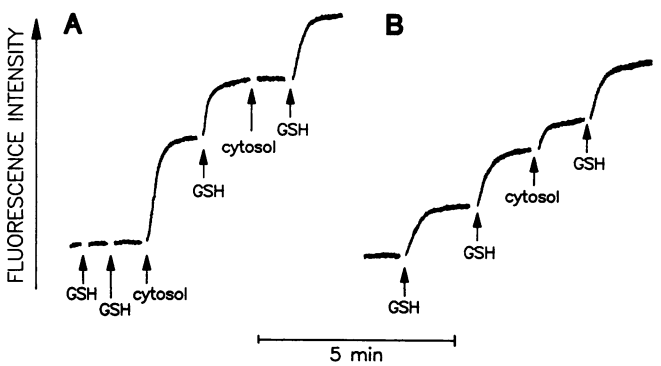

FIG. 1. Formation of fluorescent adducts between GSH and BmCl or BmBr. Fluorescence recording was done as described. The incubation medium (KTM) contained 100 μM of either BmCl (trace A) or BmBr (trace B). When indicated, GSH (5 μM, final concentration) and dialyzed cytosol (25 μg protein per ml) were added. Traces are representative of results obtained with four or more separate experiments.

of the fluorescence, characterized by a highly fluorescent region near the center of the cell (Fig. 2*a*). This distribution occurred in nearly all (>95%) cells observed. Furthermore, the regional increase in BmCl fluorescence was independent of culture conditions.

To identify the origin of the intracellular region exhibiting the highest fluorescence, BmCl-labeled hepatocytes were first treated with digitonin at a concentration that selectively permeabilizes the plasma membrane (24) and subsequently with the nuclear indicator dye Hoechst 33258. This procedure released >98% of BmCl fluorescence and revealed the localization of the nucleus (Fig. 2*b*). Comparison of BmCl- and Hoechst 33258-fluorescence images indicated a perfect identity between the regions exhibiting the highest BmCl fluorescence and those labeled with Hoechst 33258 in all cells examined (Fig. 2*c*).

To further investigate specificity of the intracellular compartmentalization of BmCl fluorescence, experiments were done to compare the regional distribution of BmCl, BmBr, and FITC fluorescence in the same cells. These indicators have been used to identify GSH, protein sulfhydryl groups, and proteins, respectively. As illustrated in Fig. 3 *a–c*, the BmCl fluorescence (*a*) exhibited a spatial distribution pattern completely different from that of BmBr (*b*) and FITC (*c*) fluorescence, indicating that a possible interference of proteins and protein sulfhydryl groups in the localization of BmCl fluorescence in hepatocytes was unlikely.

Because formation of the BmCl–GSH adduct (and hence fluorescence development) depended strictly on GSH transferase activity (ref. 19 and Fig. 1), the distribution of such activity was investigated in hepatic subcellular fractions. Table 1 shows that most transferase activity was localized in the cytosol, whereas the nuclear and microsomal fractions exhibited minor activities. This distribution was true also when transferase activity was expressed per unit volume (i.e., fluorescence arbitrary units per min per μl), which was 6.1 for nuclear fraction, 0.5 for mitochondria, and 26.2 for cytosol.

In additional experiments, hepatocytes were treated with agents known to affect intracellular GSH concentration, either by inhibiting its synthesis (buthionine sulfoximine) (5) or by promoting its consumption via oxidation (menadione) (25), alkylation (*N*-ethylmaleimide), or enzymatic conjugation [diethyl maleate (DEM)] (26). Table 2 shows that these treatments markedly decreased GSH concentration and BmCl fluorescence in freshly isolated hepatocytes as well as in 24-hr primary cultures of hepatocytes. However, in the cultured cells, the nuclear/cytoplasmic fluorescence gradient remained, although total intracellular GSH concentration was severely decreased. Furthermore, in freshly isolated as well as in cultured hepatocytes treated with relatively low DEM concentrations for a short period, BmCl fluorescence was almost completely abolished, indicating that the pool of intracellular GSH affected by these two agents was identical.

Interestingly, pretreatment of cultured hepatocytes with the uncoupler protonophore carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), induced redistribution of intracellular BmCl fluorescence from nuclei into the cytoplasm (Table 2). This effect was appreciable only after 20 min of incubation or later, when the intracellular ATP concentration had decreased to <10% of control values (data not shown).

A progressive redistribution of nuclear fluorescence occurred when BmCl-labeled cultured hepatocytes were incubated at 37°C. Fig. 4 shows that there was a continuous loss of intracellular fluorescence associated with a fluorescence increase in the extracellular medium. The loss of BmCl fluorescence was not from cell leakage, as there was no extracellular-dye uptake from the medium nor any appreciable loss of intracellular indicators (such as [2′,7′-bis(carboxyethyl)-5(6′)-carboxyfluorescein] or rhodamine 123) when

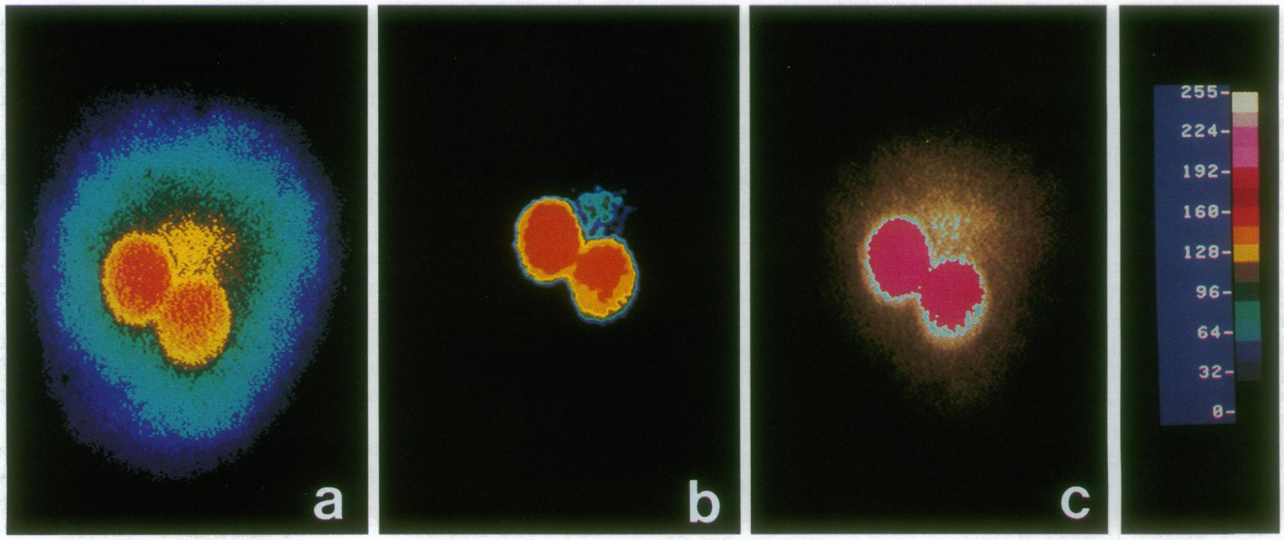

FIG. 2. Intracellular localization of GSH in hepatocytes. Twenty-four-hour cultured rat hepatocytes grown on glass coverslips were rinsed and labeled with BmCl as described. Fluorescent images of the intracellular distribution of BmCl–GSH adducts were taken before perfusion with 10 μM digitonin (*a*). Hoechst 33258 (2 μg/ml) was added, and fluorescent images showing nuclear localization of the cell previously analyzed were processed (*b*). A subsequent analysis compared the fluorescence distribution of images *a* and *b* and an identical localization of the region exhibiting the strongest BmCl–GSH fluorescence and the nucleus was observed (*c*, in violet). The images shown are representative of results obtained with 10 different experiments and were transformed using a pseudocolor scale depicted at right (arbitrary scale units).

cells were treated under the conditions noted above (data not shown). This finding suggests that the BmCl–GSH adduct was actively excreted by the hepatocytes, possibly via the transport system previously reported to be involved in excreting GSH conjugates from hepatocytes (27). Moreover, the kinetics of BmCl–GSH fluorescence decay suggest that the conjugate was first released from the nucleus into the cytoplasm and subsequently from the cytoplasm into the extracellular space.

DISCUSSION

The results reported in this study demonstrate that GSH is present at high concentrations in the nucleus of the intact hepatocyte and that a nuclear/cytoplasmic concentration gradient exists and is maintained by active mechanisms.

Several lines of evidence indicate that the fluorescence distribution in BmCl-labeled hepatocytes reflects differences in the intracellular concentration of GSH. (*i*) The reaction between the indicator dye BmCl and GSH is enzyme-mediated (19) and exhibits a strong specificity for GSH, as compared with other thiols (28, 29). (*ii*) Nuclear compartmentalization of the transferase(s) that catalyze the conjugation of BmCl with GSH is unlikely because fractionation studies showed the cytosol to be the major source of this activity. (*iii*) The finding that release of fluorescence from the nucleus into the cytoplasm, and subsequently into the incubation medium, occurs spontaneously suggests that the ad-

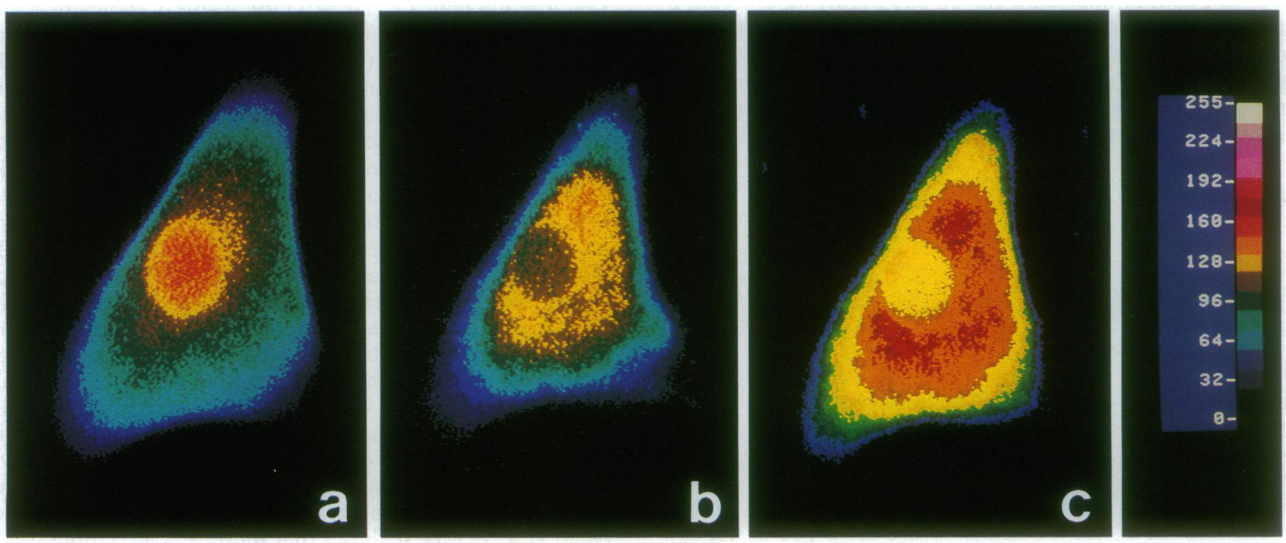

FIG. 3. Intracellular localization of GSH, protein sulfhydryl groups, and protein in hepatocytes. Twenty-four-hour cultured rat hepatocytes, grown on glass coverslips, were rinsed and labeled with BmCl as described. Fluorescent images of the intracellular distribution of BmCl–GSH adducts were then taken (*a*), and the cells were immediately fixed in ethanol/acetic acid. This treatment released all cell-associated fluorescence (data not shown). Fixed cells were subsequently incubated for 5 min with 200 μM BmBr to label protein sulfhydryl groups, and the corresponding images were processed (*b*). The hepatocytes were treated with 50 μM FITC to label protein, and the corresponding images were obtained (*c*). Images are representative of results from five different experiments and were transformed by using the pseudocolor scale depicted at right.

Table 1. Subcellular localization of enzymatic activities catalyzing the conjugation of GSH with BmCl to form a fluorescent adduct

Fraction	Reaction rate, fluorescence arbitrary units per min per mg of protein
Homogenate	70.0 ± 4.0
Nuclear fraction	15.5 ± 2.1
Mitochondrial fraction	1.5 ± 0.7
Microsomal fraction	8.0 ± 1.4
Cytosolic fraction	167.0 ± 28.0

Subcellular fractions were prepared as described. Incubations were in KMT buffer/Triton X-100/50 μM GSH/50 μM BmCl at 37°C under continuous stirring in a fluorometer cuvette. After baseline recording, the indicated fractions (50–100 μg of protein per ml) were added, and the reaction continued for 2 more min. Reaction rate was calculated by subtracting rate of fluorescence formation without the added fraction. Results are expressed as mean ± SD of three different experiments.

duct is not bound to nuclear structures. Neither does it seem probable that the fluorescent conjugate was formed in the cytoplasm and then transferred to the nucleus because time-course experiments indicated simultaneous development of fluorescence in both the cytoplasmic and nuclear compartments (data not shown). Furthermore, the uneven distribution of BmCl fluorescence in hepatocytes was not related to nonspecific subcellular modifications due to culture or incubation conditions or to a change in cell morphology with a major thickness corresponding to the nuclear region. In fact, in 24-hr-cultured hepatocytes, a depression of the cell surface occurs in proximity of the nucleus, and this effect is also evidenced by the decreased number of FITC-labeled proteins in the nuclear region. Finally, pretreatment with GSH-depleting agents abolished the formation of fluorescence in BmCl-labeled hepatocytes. Taken together, these findings provide compelling evidence that the BmCl-fluorescence

Table 2. Effects of several agents on fluorescence intensity and distribution in BmCl-loaded hepatocytes

Treatment	Hepatocytes in suspension	Adherent hepatocytes	
		Nucleus	Cytoplasm
Control	77.5 ± 5.8	158 ± 12	83 ± 9
DEM (0.1 mM)			
30 sec	31.2 ± 4.3*	ND	ND
60 sec	22.2 ± 3.8*	74 ± 11*	25 ± 6*
300 sec	2.5 ± 0.9*	12 ± 4*	8 ± 3*
BSO (0.5 mM)			
1 hr	50.2 ± 8.3*	124 ± 14*	53 ± 8*
2 hr	37.5 ± 6.6*	73 ± 8*	31 ± 5*
4 hr	16.4 ± 2.8*	ND	ND
Menadione (100 μM)			
5 min	17.5 ± 4.3*	68 ± 11*	22 ± 5*
NEM			
(25 μM) 1 min	10.1 ± 2.2*	42 ± 7*	18 ± 3*
(50 μM) 1 min	0.3*	12*	3*
CCCP (10 μM) 20 min	74.3 ± 8.2	112 ± 17*	101 ± 14

Freshly isolated rat hepatocytes in suspension (5×10^6 cells per ml) or 24-hr primary cultures of the same cell preparations were treated with the indicated agents as specified. Cells were then treated with 80 μM BmCl, and fluorescence was measured. Fluorescent images were processed, and fluorescence of the central part of the nucleus and of a portion of the cytoplasm immediately adjacent to the nucleus was measured. Results are expressed as the mean ± SD of the measurements on three different samples (for cells in suspension) or on at least 60 cells (for adherent cells). BSO, buthionine sulfoximine; NEM, *N*-ethylmaleimide; ND, not done.
*Significantly different from control, $P < 0.01$.

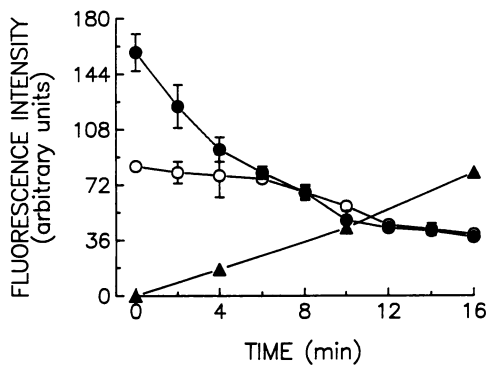

FIG. 4. Modification of fluorescence distribution in the nuclear and cytoplasmic regions of BmCl-labeled hepatocytes. Twenty-four-hr cultured rat hepatocytes, grown on glass coverslips, were rinsed and labeled with BmCl as described. After being mounted in a flow-through chamber placed on the microscope stage, the hepatocytes were continuously perfused with Krebs–Henseleit buffer maintained at 37°C. Fluorescent images of the intracellular distribution of BmCl–GSH adducts were acquired at different time points, and fluorescence of the central part of the nucleus and of a portion of cytoplasm immediately adjacent to the nucleus was measured and expressed in arbitrary units. In parallel experiments, at the indicated time points, samples of the incubation medium were taken, and the fluorescence was measured and quantitated in arbitrary units. Results are expressed as mean ± SD of 12 different experiments. ●, Nuclear fluorescence; ○, cytoplasmic fluorescence; and ▲, extracellular fluorescence.

distribution reflects differences in intracellular concentrations of GSH in hepatocytes.

The similarity in size of the GSH pools affected by DEM and labeled by BmCl raises the question of whether BmCl binds to all intracellular GSH or to only a fraction thereof. It has been suggested that the mitochondrial GSH pool is not significantly affected by low DEM concentrations (7, 30). Thus, mitochondrial GSH seems not to react with BmCl and, thus, would not contribute to the fluorescence detected in BmCl-labeled cells. This conclusion is supported by the finding that mitochondria exhibit no appreciable GSH transferase activity (cf. Table 1) and that treatment of labeled cells with 10 μM digitonin (which selectively permeabilizes the plasma membrane) released all of the intracellular fluorescence. Control experiments have shown that the fluorescent dye rhodamine 123, which actively accumulates in mitochondria, is, in fact, not released by 10 μM digitonin (ref. 24; G.B., unpublished results).

Assuming that BmCl fluorescence in labeled cells would underestimate by ≈20% the total GSH concentration and that, in hepatocytes, the cytosolic and nuclear compartments occupy a volume of 54% and 6%, respectively (31), one can calculate a nuclear GSH concentration of 19.2 mM and a cytosolic GSH level of 4.86 mM. This distribution would correspond to an "average" intracellular GSH concentration of 7.75 mM, which is very similar to that of 7.68 ± 1.22 μmol/g, reported by Meister (32).

The mechanisms involved in maintaining the high intranuclear concentration of GSH and the nuclear/cytoplasmic gradient are yet unknown. That the uncoupler CCCP collapses this gradient and releases GSH from the nucleus into the cytoplasm suggests the involvement of an energy-dependent system. In addition to depleting ATP due to mitochondrial inhibition, CCCP, by acting as a protonophore (33), may collapse transmembrane potential maintained by proton gradients. However, this mechanism is probably not the one operative in the CCCP-induced dissipation of the nuclear/cytoplasmic GSH concentration gradient. In fact, the protonophoric activity of CCCP occurs in <1 min (33), whereas the effects of CCCP on GSH distribution became

apparent only after 20 min of incubation, at which time the intracellular ATP concentration had decreased to <10% of the control value.

It has long been thought that the existence of pores in the nuclear envelope prevents any selective compartmentalization of small molecules and ions in the nuclear matrix (34). However, this view has been shown to be incorrect, as active transport systems for ions have been detected in the nuclear membrane (35) and nuclear/cytoplasmic ion gradients exist in living cells (36, 37) It is, therefore, conceivable that the transport of GSH between cytoplasm and nucleus is also actively regulated.

The presence of high concentrations of GSH in the nucleus of hepatocytes would be important to the several GSH-dependent enzymes associated with nuclear structures. Ketterer and associates (38) have recently reported the occurrence of GSH transferases in the nuclear matrix of hepatocytes, which would participate in the metabolism of lipid hydroperoxides and in DNA repair. Moreover, reports from Reed and coworkers (39, 40) have demonstrated that lipid peroxidation may occur in isolated liver nuclei and that it can be efficiently prevented by GSH-dependent enzyme systems. Finally, a nuclear and perinuclear localization of the phospholipid hydroperoxide GSH peroxidase has been recently discovered (41). In addition to its participation in various detoxication reactions, GSH is critically involved in many physiological processes, including the regulation of nuclear matrix organization (42); the maintenance in the reduced state of the cysteine residues essential for the zinc finger structural motif (43) and other cysteine-rich motifs (44) of DNA-binding proteins; and the regulation of chromatin compaction (45) and DNA synthesis (46), as well as the activation of nuclear factors and transcription of human immunodeficiency virus (47). The demonstration of high concentrations of GSH in the nucleus strengthens the importance of this tripeptide in the reactions described above and encourages further investigation of the biochemical mechanisms involved in regulating this compartmentalization process.

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