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THE S-THIOLATION OF HEPATOCELLULAR PROTEIN THIOLS DURING DIQUAT METABOLISM

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Abstract—The effects of diquat metabolism on the protein thiol (PrSH) status of *bis*-chloronitrosourea-pretreated hepatocytes have been studied. Using a conventional, dithionitrobenzene-based assay for free PrSH in trichloroacetic acid-precipitated protein, control levels of PrSHs (83 ± 6 nmol/mg protein) were unaltered during the initial 60 min of incubation of the cells with 1 mM diquat. However, using a radiochemical method for the determination of glutathionylation of PrSH [Grimm *et al.*, *Biochim Biophys Acta* **844**: 50–54, 1985], in which the hepatocytes were prepared from diethylmaleate-pretreated animals and reloaded with reduced glutathione (GSH) in the presence of [^{35}S]methionine and cycloheximide, oxidation of hepatocellular PrSH by stimulated S-thiolation with GSH could be demonstrated. The S-glutathionylation of the protein was maximal after 30 min of treatment of the cells and preceded the onset of membrane leakage. However, the quantity of GSH mixed disulfide formed was limited to a maximum of 1.4 ± 0.4 nmol GSH/mg protein, indicating the oxidation of only 2% of the total hepatocellular PrSH by S-thiolation. This percentage depletion is below the working variability of the colourimetric PrSH assay utilized and indicates strongly the use of the S-thiolation assay in the study of the possible effects of other redox-cycling cytotoxins on cellular PrSH status, as these may not be evident with conventional spectrophotometric techniques. The analysis of the cellular protein from diquat-treated cells by SDS–PAGE and autoradiography indicated the S-glutathionylation of a variety of cellular proteins, including species with molecular masses 17, 24, 26, 30, 40, 43 and 46 kDa. Although the identities of these species are uncertain (the 30-kDa protein may be equivalent to carbonic anhydrase as reported by Rokutan *et al.*, *Biochemistry* **179**: 233–239, 1989), it may be that oxidative modification of these proteins by stimulated S-glutathionylation may be an important early event in the mechanism of the hepatotoxicity of diquat.

The bipyridylium herbicide diquat induces acute hepatocellular toxicity in association with the production of reactive oxygen metabolites by redox cycling [1]. This toxicity is closely correlated with the accumulation of oxidized glutathione (GSSG†) in the cells and is, indeed, only manifested when GSSG reductase is inhibited by *bis*-chloronitrosourea (BCNU) [2, 3]. Despite these observations within the soluble thiol pool of the cells, little or no information is available concerning potential effects of diquat-induced oxidative stress on protein thiol (PrSH) components of the cells.

Most cellular proteins contain cysteinyl thiols which are involved in diverse functions such as the control of protein folding, the association of protein subunits into oligomeric structures and the catalytic function of enzymes [4]. Post-translational modification of PrSHs may alter substantially the function of the proteins concerned. Such modifications may be induced by xenobiotics in a variety of ways. Reactive, electrophilic species may form covalent adducts with PrSHs and such alkylations have been implicated in the mechanisms of toxicity of paracetamol [5] and menadione [6]. Alternatively, the redox status of PrSHs can be altered either by direct oxidation or by S-thiolation of PrSHs by thiol–disulfide exchange (TDE) reactions with low molecular mass thiol redox couples such as GSH/GSSG [7]. Clearly, such oxidative/S-thiolative modification of hepatocellular protein is indicated strongly during the metabolism of diquat.

Previous reports have demonstrated a 10, 15 and 20% depletion of PrSHs during diquat metabolism in BCNU-pretreated hepatocytes over the initial 30, 60 and 90 min of metabolism, respectively [8]. A conventional assay of the reactivity of trichloroacetic acid(TCA)-precipitated cellular protein with dithionitrobenzene (DTNB) was employed in these studies, which presents several draw-backs to the interpretation of the data. These include the possibility of artefacts during sample preparation, the inability to monitor PrSH heterogeneity in complex protein mixtures and the inherent variability of the assay. In addition, these authors did not define the mechanism of the depletion of PrSHs. However, as GSH levels are depleted by over 80% after 5 min of incubation, with a corresponding rapid accumulation of GSSG [2, 8], TDE reactions between GSSG and PrSHs may be involved.

In the present study we failed to detect significant depletion of hepatocellular PrSHs in BCNU-pretreated hepatocytes during diquat metabolism using the conventional DTNB assay for PrSHs. However, using a modification of the technique described by Grimm *et al.* [9]—in which the cellular GSH pool is depleted *in vivo* by diethylmaleate (DEM)-pretreatment of rats, and hepatocytes are

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† Abbreviations: GSH, reduced glutathione; GSSG, oxidized glutathione; PrSH, protein thiol; OTNB, dithionitrobenzene; BCNU, *bis*-chloronitrosourea; TDE, thiol–disulfide exchange; TCA, trichloroacetic acid; DEM, diethylmaleate; mBBr, monobromobimane.

then prepared, treated with BCNU, washed and the GSH pool resynthesized in the presence of [^{35}S]-methionine and the protein synthesis inhibitor cycloheximide—we have demonstrated rapid, but quantitatively limited, S-glutathionylation of hepatocyte PrSHs during diquat redox cycling. Additionally, the technique allowed the determination of the heterogeneity of S-thiolation, revealing the molecular masses of several S-thiolated protein species. Taken together, these data indicate that post-translational modification of hepatocyte protein by stimulated S-glutathionylation may be an important, early event in the molecular mechanism of toxicity of diquat in this system. Additionally, the data indicate that the S-glutathionylation assay should be used for the determination of PrSH depletion in cases where the toxicity of molecules, particularly redox cyclers, is thought to involve oxidation of PrSHs which might not be detectable using other less sensitive assays.

MATERIALS AND METHODS

Chemicals. L-[^{35}S]methionine (sp.act. 1245 Ci/mmol) was purchased from New England Nuclear (Du Pont Scandinavia AB, Stockholm, Sweden). Diquat dibromide (>99%) was the kind gift of Dr L. L. Smith (Imperial Chemical Industries, London, U.K.). BCNU was from Bristol-Meyers Pharmaceuticals (Stockholm, Sweden) and DEM, DTNB, cycloheximide, puromycin and chloramphenicol were all purchased from the Sigma Chemical Co. (St Louis, MO, U.S.A.). Monobromobimane (mBBr) was from Calbiochem (La Jolla, CA, U.S.A.). All other chemicals were obtained from local suppliers in the highest grade available.

Preparation of hepatocytes. Male Wistar rats (230–250 g), allowed food and water *ad lib.*, were used throughout the study. Animals were pretreated with DEM(3.9 mmol/kg body wt in corn oil, i.p.) 60 min prior to the preparation of hepatocytes by the collagenase perfusion technique of Moldéus *et al.* [10]. Hepatocytes (average 450×10^6 cells/liver) were then suspended in Krebs–Henseleit buffer supplemented with 12.5 mM HEPES, pH 7.4, which was used for all incubations unless otherwise stated, and assayed for their GSH content and viability as described below. All subsequent incubations were performed in rotating, round-bottomed flasks under a carbogen atmosphere.

Inhibition of GSSG reductase, labelling of cellular GSH and incubation with diquat. Hepatocyte GSSG reductase was inhibited in DEM-pretreated hepatocytes by the incubation of the cells (10^8 cells at 2×10^6/mL) with BCNU (50 μM) for 20 min. The cells were then washed twice with incubation buffer, aliquots were removed for the determination of cell viability, and GSH content, and the cell batch was then incubated with an amino acid medium supplemented with [^{35}S]methionine (0.2 mM, 55 μCi/cell batch) and cycloheximide (0.5 mM) for 90 min. The cells were then washed four times in incubation buffer, resuspended at 10^6 cells/mL and assayed for cell viability, GSH levels and specific activity, and the back-ground radioactivity in protein, all as described below.

Incubations were then started by the addition of diquat (1 mM) and aliquots were removed for the determination of cell viability, GSH levels, PrSHs and GSH–protein mixed disulfides as described below.

Biochemical analysis. Cell viability was determined by the method of Trypan blue (0.16%) exclusion. Cellular GSSG reductase activity was assessed as described previously [2, 3] and protein determined by the method of Peterson [11].

GSH in cellular aliquots (0.1 mL, 10^5 cells/assay) was determined using *in situ* derivatization of the cells with mBBr and subsequent separation and quantitation of the mBBr–GSH adduct by HPLC/fluorescence detection as described previously [12]. Because of the quantitative recovery of GSH as the mBBr adduct, collection of the mBBr–GSH adduct and quantitation of the radioactivity associated with this peak by liquid scintillation spectrometry facilitates the determination of the specific activity of the GSH.

Bulk cellular PrSHs were determined by the reaction of TCA-precipitated and washed cellular protein (from 1-mL aliquots, 10^6 cells/assay) with DTNB as described previously [6]. Control and diquat-treated cells were analysed for protein–GSH mixed disulfides essentially as described previously [7, 9]. Briefly, aliquots (2 mL, 2×10^6 cells/assay) were rapidly mixed 1:1 with 10% perchloric acid containing 100 mM *N*-ethylmaleimide. The resultant protein pellet was washed three times with 5% PCA, 50 mM *N*-ethylmaleimide and dissolved in 2% SDS with neutralization with $NaHCO_3$. Aliquots of this were assayed for protein content and total radioactivity. Other aliquots were applied directly to 12% SDS–PAGE gels [13], separated and subjected to auto-radiography using Kodak Tri-pan-X X-ray film and scintillant enhancement. In some cases the protein was reduced with 1% mercaptoethanol (5 min, 90°) prior to electrophoresis.

Statistical analysis. Groups of data were compared for significant differences using the Student's *t*-test for paired observations.

RESULTS

The data shown in Table 1 indicate the effect of various pretreatments of the hepatocytes used in the present investigations. It can be seen that hepatocytes prepared from DEM-pretreated rats contained 25% (14 ± 4 nmol/mg protein) of the control levels of GSH whilst their cell viability and GSSG reductase activity were unaltered as compared with controls. Further treatment of these cells with BCNU resulted in a greater than 95% inhibition of GSSG reductase and concurrent decreases in cell viability (to 69 ± 5% of total cells) and cellular GSH (to 8 ± 1 nmol/mg protein). After washing these cells and incubation in a medium containing amino acids, cellular GSH levels recovered to >90% of control levels (to 44 ± 6 nmol/mg protein) and cell viability was recovered to 77% of control cells, without affecting GSSG reductase activity. The inclusion of [^{35}S]methionine and cycloheximide in this recovery buffer did not influence the parameters tested (N = 3 on all data above).

Figure 1 shows that when either control cells or

Table 1. Cell biochemical parameters from the preparation of BCNU-pretreated, [^{35}S]GSH-labelled hepatocytes

Cell preparation	Cell viability* (% total)	GSSG reductase† activity (% control)	GSH levels‡ (nmol/mg)
Control	95 ± 2	100	48 ± 3
DEM	93 ± 3	94 ± 3	14 ± 4
DEM/BCNU	69 ± 5	4 ± 1	8 ± 2
DEM/BCNU AA	77 ± 4	5 ± 1	44 ± 6
DEM/BCNU AA/cycloheximide/^{35}S	82 ± 6	6 ± 1	42 ± 4 ($9.3 \pm 1.6 \times 10^3$)§

* Cell viability was assessed by Trypan blue exclusion, †GSSG reductase activity was assayed spectrophotometrically by following the oxidation of NADPH in the presence of GSSG and ‡cellular GSH was assayed with monobromobimane, all as described in Materials and Methods.

§ The specific activity of the resultant [^{35}S]GSH (dpm/nmol). AA, the amino acid medium used in the recovery of BCNU-pretreated cells. All of the data are means ± SEM, N = 3.

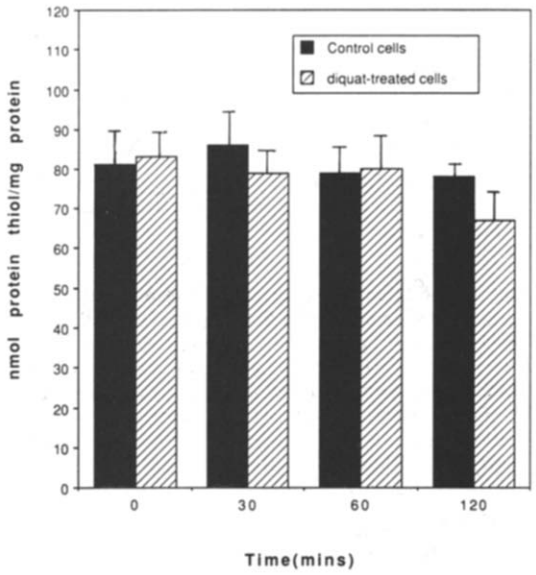

Fig. 1. The effect of diquat metabolism on total PrSHs in BCNU-pretreated hepatocytes. Freshly-isolated hepatocytes were pretreated with BCNU and allowed to recover their cellular viability and GSH levels in an amino acid medium as described in Materials and Methods. Control (inhibited GSSG reductase) cells (10^6/mL, in Krebs–Henseleit buffer, pH 7.4) and similar cells treated with diquat (1 mM) under a carbogen atmosphere at 37° were then assayed for PrSHs using the reaction of DTNB with TCA-precipitated cell protein as described in Materials and Methods. The data are means ± SEM, N = 4. No significant differences were detected between the control and diquat-treated cells.

BCNU-pretreated, GSH-repleted cells were treated with diquat (1 mM) for up to 2 hr, no significant depletion of bulk PrSH (from 83 ± 6 nmol PrSHs/mg protein, N = 3) could be detected using the reaction of TCA-precipitated cell protein with BCNU.

The success of the methodology for the determination of the formation of GSH–protein mixed disulfides relies on a number of parameters. Firstly, the data in Table 1 indicates that the inclusion of cycloheximide (500 μM) in the recovery buffer did not affect the ability of the cells to resynthesize GSH, nor did it affect cell viability or GSSG activity. Secondly, control experiments indicated that when cyclohexmide was excluded from the radiolabelled recovery buffer cellular protein was labelled at a rate of $12.3 \times 10^3 \pm 560$ dpm/mg protein/hr (N = 3). The inclusion of cycloheximide (500 μM) in the recovery buffer reduced this incorporation by >90%, an inhibitory effect which was not potentiated by the co-inclusion of either of the mitochondrial protein synthesis inhibitors puramycin and chloramphenicol (100 μM each, data not shown).

The incubation of diquat with BCNU-pretreated, [^{35}S]GSH-labelled cells in the presence of cycloheximide caused a rapid and significant increase in the amount of radioactivity associated with hepatocyte protein (Fig. 2). The binding of GSH was maximal after 60 min of incubation and amounted to 1.4 ± 0.4 nmol GSH/mg protein (N = 3). The pretreatment of the protein with 1% mercaptoethanol, for 5 min at 90°, removed 95% of the bound radioactivity above background from the protein at all time points (data not shown). Fig. 3 shows that the S-thiolation of bulk protein followed the rapid depletion of cellular GSH, which was maximal after 15 min of incubation, but preceeded the onset of membrane leakage.

When the protein was subjected to separation by SDS–PAGE it can be seen in Fig. 4 that several protein species were shown to be labelled, particularly species with molecular masses 17, 24, 26, 30, 40, 43 and 46 kDa. It can be seen that the labelling of all of these bands increased uniformly with time (Fig. 4, lanes 1–6), in parallel with the labelling kinetics of the bulk protein (Fig. 2). Figure 4 (lane 7) shows that the preincubation of the protein with 1% mercaptoethanol for 5 min at 90° removed the radioactivity from the protein. Control protein from untreated cells was not labelled at any time point (Fig. 4, lane 8).

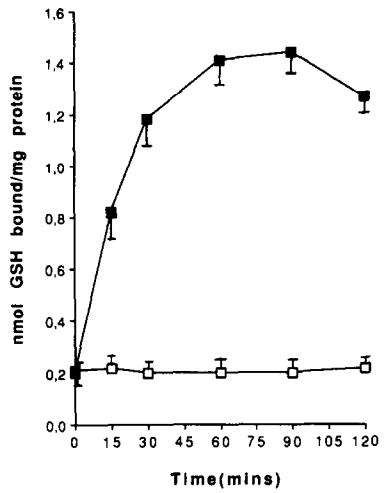

Fig. 2. The formation of GSH–protein mixed disulfides as a result of diquat metabolism in BCNU-pretreated and [^{35}S]GSH-labelled hepatocytes. Rats were pretreated with DEM and GSH-depleted hepatocytes prepared. These cells were then treated with BCNU and allowed to recover viability in an amino acid medium supplemented with cycloheximide and [^{35}S]methionine, washed and the specific activity of the cellular GSH determined, all as described in Materials and Methods. The cells (10^6/mL in Krebs–Henseleit buffer, pH 7.4) were then incubated in the absence (□) or presence of diquat (1 mM) (■) and aliquots (2 mL) taken periodically for the assay of bulk GSH–protein mixed disulfides following TCA precipitation and washing of the protein, dissolution in SDS and assay of the ^{35}S of the protein by liquid scintillation counting, all as described in Materials and Methods. The data are means ± SEM, N = 3 and all data obtained from the diquat-treated cells are significantly ($P \leq 0.01$) different from the controls.

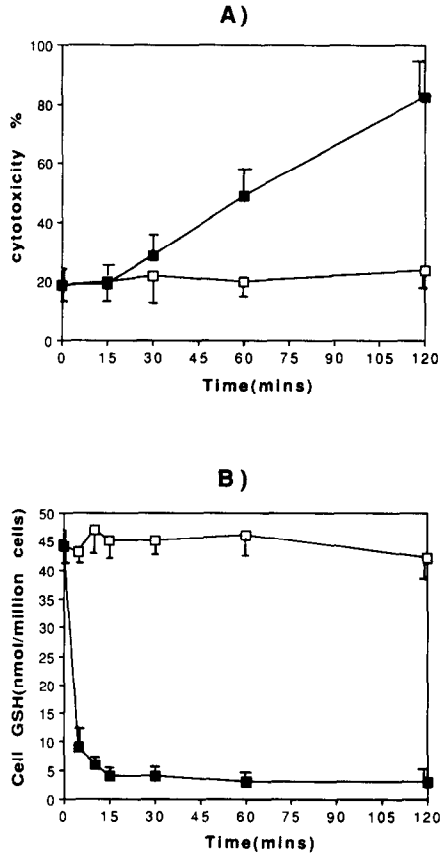

Fig. 3. The effects of diquat metabolism on the viability (A) and GSH content (B) of BCNU-pretreated and [^{35}S]-GSH-labelled hepatocytes. The cells were prepared and treated as described in the legend to Fig. 2. The cellular viability was assessed by the exclusion of Trypan blue and the GSH content by derivatization of the cells with mBBr and subsequent HPLC analysis, both as described in Materials and Methods. The data are means ± SEM, N = 3. (□) Control cells, (■) diquat (1 mM)-treated cells.

DISCUSSION

Although the hepatocellular toxicity of diquat is clearly dependent on the induction of oxidative stress associated with the rapid depletion of GSH [2, 3] and ascorbate [14], there is little appreciation of the consequences of an "overflow" of reactive oxygen metabolites on structural components of cells. It is clear that diquat induces lipid peroxidation [7], but that this generally coincides with the onset of Trypan blue leakage and may be temporally uncoupled from toxicity by coincubation with antioxidants [2, 15]. This suggests that lipid peroxidation does not play a major causative role in the diquat mechanism of toxicity.

In the present paper we have concentrated on the possible oxidative effects of diquat-induced redox cycling on hepatocellular protein. Using the conventional assay of PrSHs in bulk cellular proteins using the reaction of TCA-precipitated protein with DTNB, we were unable to demonstrate a significant oxidation of PrSHs during diquat metabolism before the onset of considerable membrane leakage. Although the control levels of PrSHs are similar to those reported previously by Sandy *et al.* [8], these authors demonstrated a 12 and 20% depletion of PrSHs after 30 and 90 min of incubation of BCNU-pretreated hepatocytes with diquat, respectively. One reason for the discrepancy in the data may lie in the fact that these authors used 1.5 mM diquat in their experiments and not the 1 mM employed in the present experiments. Additionally, the SEM values reported in the present studies (±5% to ±9%) are somewhat higher than those reported by Sandy *et al.* [8].

In contrast to results obtained with the above, relatively crude, technique, we were able to quantify significant oxidation of PrSHs by stimulated S-thiolation of the protein with GSH during the metabolism of diquat. These results were obtained by a modification of the technique used by Grimm *et al.* [9]. Although this approach has been applied recently to cultured hepatocytes [7], there has been no report of its use in suspensions of isolated hepatocytes. In order to achieve considerable labelling of the cellular GSH pool with [^{35}S]methionine without labelling of the cellular protein by *de novo*

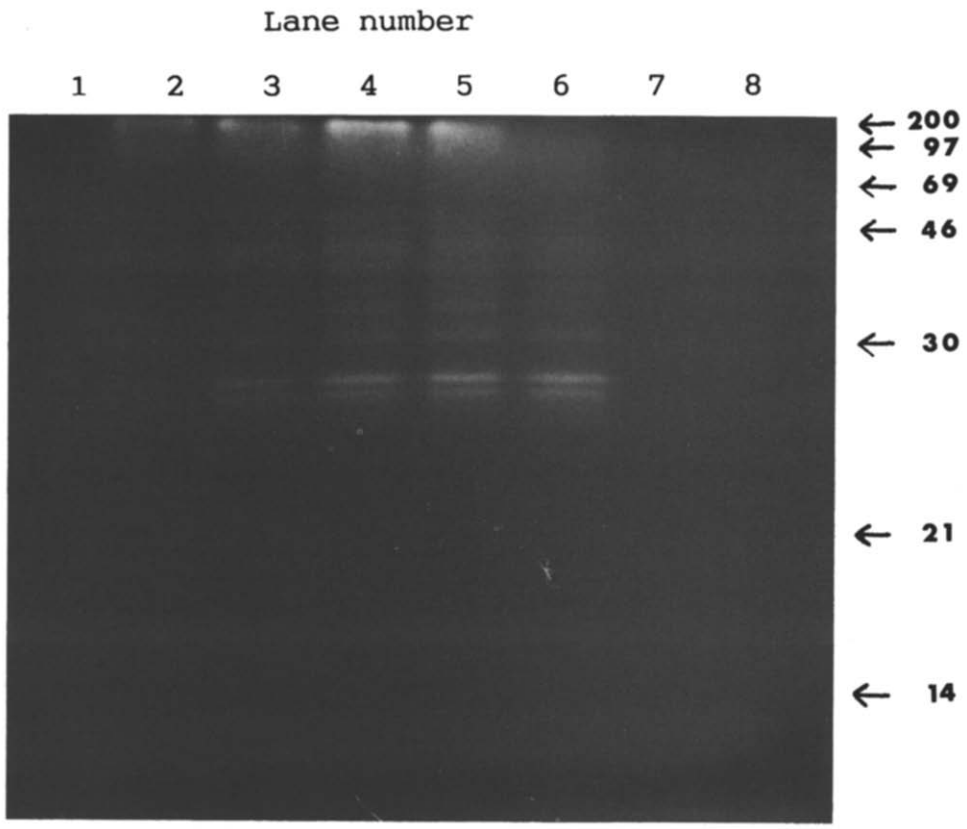

Fig. 4. The analysis of diquat-induced protein S-glutathionylation in BCNU-pretreated and [^{35}S]GSH-labelled hepatocytes by SDS-PAGE. The cells were prepared and treated with diquat (1 mM) as described in the legend to Fig. 2 and, following TCA precipitation and washing, the SDS-solubilized fraction was subjected to SDS–PAGE on 12% acrylamide gels under non-reducing conditions and autoradiographed, all as described in Materials and Methods. Lanes 1–6: BCNU-pretreated, diquat-treated cells at 0, 15, 30, 60, 90 and 120 min, respectively. Lane 7: as lane 6 but the protein was pretreated with 1% mercaptoethanol for 5 min at 90° before application to the gel. Lane 8: similar cells after 120 min in the absence of diquat. Gel tracks were loaded with 100 μg protein.

synthesis, it was necessary to deplete hepatocyte GSH by pretreatment of the rats with DEM prior to the preparation of the cells (Table 1). In agreement with the previous data [2], the treatment of these cells with BCNU further depletes cellular GSH and reduces the viability of the cell batch (Table 1). Again, in agreement with previous data [2, 3], near control levels of GSH could be restored in the cells by their incubation in an amino acid-supplemented buffer. The inclusion of [^{35}S]methionine in this recovery buffer, along with cycloheximide (500 μM), did not affect the synthesis of GSH but resulted in considerable labelling of this thiol pool without appreciable labelling of the protein.

Figure 3 confirms that the kinetics of GSH depletion and onset of membrane leakage caused by diquat metabolism in BCNU-pretreated hepatocytes in the present experiments are similar to those reported previously [2, 3]. Figure 2 shows that maximal levels of GSH–protein mixed disulfides are reached before the onset of membrane leakage. Due to the integral involvement of thiol groups in the function of many proteins, this might indicate that these redox events on cellular proteins might be important in the mechanism of diquat cytotoxicity. Several mechanisms may be operating to link the depletion of GSH and the formation of mixed disulfides. Other authors have suggested that TDE reactions between PrSHs and GSSG are of importance. In the present experiments we confirmed a rapid depletion of GSH from the cells, which was maximal after 15 min of metabolism (Fig. 3). These kinetics and the extent of depletion (90%) also agree well with previous data [2, 3]. It is known that this depletion of GSH is rapidly paralleled by a near quantitative increase in GSSG in the cells which is maximal after 15 min of incubation [2, 3]. By comparing these data to the kinetics of mixed disulfide formation detected here it is apparent that the glutathionylation of hepatocyte protein closely follows the oxidation of GSH in the cells, suggesting a dependence on intracellular GSSG concentration [7, 9]. However, it is uncertain from the present data whether the mixed disulfides are formed purely

chemically in this system, or if thiol transferase enzymes are involved. Similarly, it must be born in mind that diquat may stimulate the formation of GSH–protein mixed disulfides by processes which are not dependent on TDE between GSSG and PrSHs. Thus, diquat may stimulate direct oxidation reactions between GSH and PrSHs [7].

It is evident from the present results that only a small fraction (*ca.* 3%) of the cellular GSH which is depleted from the hepatocytes during diquat metabolism optimally resides on the protein in mixed disulfides. Previous work be Bellomo *et al.* [16] had demonstrated that considerably higher levels of GSH–protein mixed disulfides resulted from the metabolism of menadione in control hepatocytes, where 4–5 nmol GSH were detected bound to protein using reductive cleavage of the protein and quantitation of the GSH released [16]. The quantitative discrepancies between the effects of diquat and menadione may be due to either the different analytical approaches employed or the fact that diquat experiments are performed in BCNU-pretreated cells. On the other hand, it is unlikely that the absolute intracellular GSSG concentrations in the respective systems play a major role. Menadione depletes hepatocyte GSH by the formation of conjugates and by oxidation go GSSG via redox cycling [16]. Thus, the intracellular concentration of GSSG in menadione-treated cells is actually less than in diquat-treated cells where only the oxidation of GSH only occurs. Irrespective of these discrepancies it is evident, in terms of the total PrSH available, that hepatocytes sustain relatively low levels of GSH–protein mixed disulfides as compared with other cell types such as human endothelial cells [17].

In comparing the two techniques used in the present investigation to quantitate diquat-induced depletion of hepatocyte PrSHs it is evident that, within the accepted errors of the DTNB technique (±5%), it would not be possible to detect any depletion of PrSHs by S-glutathionylation as a result of diquat metabolism. Thus, this indicates strongly the use of the S-thiolation assay in the study of the potential effects of other redox cyclers on cellular PrSHs as small, but critical, depletions may be missed using conventional DTNB-based methods.

Apart from the inherent sensitivity of the S-thiolation assay, the technique allows for the identification of protein species sustaining mixed disulfides with GSH [7, 9]. It is clear from Fig. 4 that diquat metabolism caused the S-glutathionylation of several discrete protein bands on SDS–PAGE gels. The identities of these protein substrates are as yet unknown, but there are some similarities with previous results in cultured hepatocytes. For instance, Rokutan *et al.* [18] have reported the diamide- and *tert*-butyl hyderoperoxide-stimulated S-thiolation of a 30-kDa substrate, similar perhaps to the 30-kDa species detected in the present work, which they identified as carbonic anhydrase [18]. Thomas *et al.* [7] have demonstrated the S-thiolation of substrates with molecular masses of 28, 40, 43, 46, 52 and 72 kDa in oxidant-treated hepatocytes. It may be that several of these, particularly the 40-, 43- and 46-kDa species, are similar to the 39-, 42- and 45-kDa species S-thiolated by diquat metabolism. Similarly, the 17-kDa species which is S-thiolated during diquat metabolism may be microsomal GSH transferase, an abundant microsomal protein (3% of the total hepatocyte microsomal protein) which possesses one free thiol group which is sensitive to alkylation/acrylation reactions [19].

Despite these observations, until the identities of the S-glutathionylated species are firmly established a definitive role for redox changes to cellular PrSHs in the molecular mechanism of diquat cytotoxicity cannot be established firmly. These aspects of the effects of diquats on hepatocyte protein ontogeny are the subject of further studies.

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