Biochemical Pharmacology, Vol. 42, Suppl., pp. S197–S207, 1991.
Printed in Great Britain.

0006-2952/91 $3.00 + 0.00

A COMPARISON OF PROTEINS S-THIOLATED BY GLUTATHIONE TO THOSE ARYLATED BY ACETAMINOPHEN*

RAYMOND B. BIRGE,†‡ JOHN B. BARTOLONE,†§ STEVEN D. COHEN,‖
EDWARD A. KHAIRALLAH†¶ and LORI A. SMOLIN†

Departments of †Molecular and Cell Biology and ‖Pharmacology and Toxicology,
University of Connecticut, Storrs, CT 06269-3125, U.S.A.

(*Received* 30 *October* 1990; *accepted* 26 *June* 1991)

Abstract—This study was designed to evaluate whether the same proteins that irreversibly bind reactive electrophiles of drugs also bind glutathione (GSH) under oxidative conditions. Specifically, proteins that can be arylated by acetaminophen were compared to those that form glutathione-protein mixed disulfides (PSSG) after incubation with diamide. Data are presented which suggest that both GSH and acetaminophen bind to a subset of *N*-ethylmaleimide (NEM)-reactive protein thiols. To evaluate the pattern of proteins that bind GSH, PSSGs were formed *in vitro* by incubating cytosolic proteins with GSH and diamide. A sensitive procedure was developed in which PSSGs were first reduced with 0.1 mM dithiothreitol (DTT), and the newly exposed protein thiols were labeled with either [^{3}H]NEM (for quantitative analysis) or with fluorescein-5-maleimide (for visual detection). Acetaminophen binding was achieved by incubating cytosolic proteins *in vitro* with the reactive acetaminophen metabolite, *N*-acetyl-*p*-benzoquinoneimine (NAPQI). Proteins from both assays were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to nitrocellulose for Western blot analysis. Acetaminophen binding was detected by immunoblotting with an affinity-purified antibody against acetaminophen, and PSSGs were visualized using anti-fluorescein antibodies. In both instances, binding to proteins was observed to be selective. A comparison of the proteins modified by GSH binding with those that bind acetaminophen indicates that the major cytosolic acetaminophen-binding protein of 58 kDa may also be modified by glutathiolation under oxidative conditions.

Cysteine residues of proteins are involved in a number of reactions that may affect protein structure and function. The metabolites of many drugs are known to interact with protein thiols [1–4], and enzyme activity can be modified by binding low molecular weight thiols and disulfides, such as gluthatione (GSH) [5–8]. Covalent modifications of cysteinyl residues by either mixed disulfide formation or by drug arylation, therefore, could alter many aspects of protein structure and function and, ultimately, cell viability. Not all cysteine residues in proteins, however, are equally susceptible to covalent modifications [5, 8–10]. At physiological pH only the protein thiols that have low pK_a values are deprotonated to a thiolate anion and, as such, can become nucleophilic targets [8]. Likewise, only proteins with exposed cysteine residues are likely to be modified by thiol–disulfide interchange [11, 12] or by reactive intermediates [1–4].

Acetaminophen (paracetamol, 4′-hydroxyacetanilide, *N*-acetyl-*p*-aminophenol), a widely used analgesic, can be oxidized by cytochrome P450 to *N*-acetyl-*p*-benzoquinoneimine (NAPQI) [13], a reactive metabolite that possesses both electrophilic and oxidative properties [14, 15]. As an electrophile, NAPQI can covalently bind to cysteine residues in GSH or proteins. As an oxidizing agent, NAPQI may also oxidize GSH or protein thiols. Even though both properties may contribute to the cytotoxic actions of NAPQI [15, 16], immunochemical analysis of acetaminophen covalent binding to proteins has demonstrated that only a few proteins become selectively targeted [17–25] and the arylation of these proteins, especially the 58-kDa target, has been correlated with the ensuing hepatotoxocity [17, 21–24].

Glutathione-protein binding has also been demonstrated to be selective in experiments in which cardiac, liver or lung cells were exposed to diamide or hydroperoxide [26–32]. However, the detection of PSSG is problematic. One method utilized an incubation of cells with cycloheximide during equilibration with [^{35}S]cysteine [26]. This permits incorporation of the radiolabeled cysteine into GSH and not into proteins and allows the PSSG to be detected by autoradiography of the electrophoretically resolved proteins. The need to inhibit protein synthesis by cycloheximide limits the usefulness of this method in many experimental situations and may itself alter the formation of mixed disulfides. A second method of detecting PSSG

* Presented in part at the annual meetings of the American Society of Cell Biology (*J Cell Biol* **107**: 635a, 1988; and *J Cell Biol* **111**: 59a, 1990) and of FASEB (*FASEB J* **3**: A1265, 1989).

‡ Present address: Department of Molecular Oncology, The Rockefeller University, 1230 York Ave, New York, NY 10021-6399.

§ Present address: Richardson Vicks, Inc., 1 Fall Mill Crossing, Shelton, CT 06484.

¶ Correspondence: Dr. Edward A. Khairallah, Department of Molecular and Cell Biology, U-125, University of Connecticut, 75 North Eagleville Road, Storrs, CT 06269-3125.

formation assumes that changes in the pI of S-thiolated proteins will alter their migration upon thin-gel isoelectric focusing [27]. Proteins modified by binding GSH can be identified by comparing their electrophoretic migrations to that of unmodified proteins. Since the proteins must be detected by densitometric scanning of stained gels, proteins present in low abundance in complex tissue samples may be difficult to detect by this method.

Despite the evidence that covalent modifications of cysteinyl residues by either mixed disulfide formation or by drug arylation may alter many aspects of protein structure and function and ultimately cell viability, no one to date has attempted to evaluate whether the specific proteins which bind electrophiles are also susceptible to oxidation. Since many toxic agents that covalently bind to protein thiols are also potentially capable of oxidizing cysteine residues [15, 16, 33], this study was undertaken to compare *in vitro* the proteins that bind the reactive metabolite of acetaminophen to those that bind GSH following treatment with diamide. Towards this goal we report the development and characterization of a sensitive procedure to detect specific proteins that can form PSSG. The present study provides evidence that some of the cytosolic proteins targeted by electrophiles, including the 58-kDa acetaminophen-binding protein, are also susceptible to glutathiolation under *in vitro* oxidative conditions.

MATERIALS AND METHODS

Materials. Radioactive *N*-ethylmaleimide (NEM), labeled with either ^{3}H or ^{14}C, [^{3}H]GSH (reduced form), uniformly labeled [^{3}H]acetaminophen, ^{125}I-conjugated goat anti-rabbit IgG, and En^{3}Hance were obtained from Dupont New England Nuclear (Boston, MA). The tissue solubilizer, NCS, was obtained from Amersham-Searle (Arlington Heights, IL). Fluorescein-5-maleimide was purchased from Molecular Probes (Portland, OR) and rabbit anti-fluorescein antibodies were obtained from Chemicon International (El Segundo, CA). Electrophoretic grade Tris–HCl, glycine, acrylamide, and *N,-N'*-methylene bis acrylamide were purchased from ICN Biochemicals (Cleveland, OH). Nitrocellulose membranes (0.2 μm) were purchased from Schleicher & Schuell (Keene, NH). Sephadex G-25 (fine) was obtained from Pharmacia (Piscataway, NJ). Filtration discs (0.22 μm) were purchased from Micro Separations Inc. (Westborough, MA). All other reagents were purchased from the Sigma Chemical Co. (St. Louis, MO).

Mouse liver cytosolic proteins. Livers were obtained from 3- to 4-month-old Crl:CD-1 male mice (Charles River, Wilmington, MA) maintained on Purina rat chow *ad lib.* Animals were housed three per cage and maintained on a 12-hr light–dark cycle with constant temperature and humidity control. Mice (averaging 35 g) were killed by cervical dislocation. Livers were homogenized in 3 vol. of ice-cold buffer containing 0.25 M sucrose, 10 mM Tris–HCl, 1 mM $MgCl_2$, pH 7.4 (STM), and the cytosol was obtained by centrifugation at 105,000 *g* for 60 min.

Assay of protein thiols. Protein thiols that are accessible under non-denaturing conditions at pH 7.4 were assayed by monitoring the binding of radiolabeled NEM [33, 34]. To quantify binding, cytosolic proteins were incubated at 4° with up to 10 mM [^{3}H]NEM or [^{14}C]NEM (0.1 mCi/mmol) for the times indicated. Proteins were precipitated with an equal volume of 2 N perchloric acid (PCA). After standing on ice for 5 min, the solutions were centrifuged for 5 min at 10,000 *g* and the protein pellets were washed three times with 80% methanol containing 10 mM unlabeled NEM, and once with 1 N PCA. Samples were solubilized in NCS and counted in a Beckman model 3801 liquid scintillation counter.

To determine the pattern of proteins that bind NEM at pH 7.4 under non-denaturing conditions, cytosolic proteins were first reacted with 10 mM [^{3}H]NEM (50 mCi/mmol). Prior to separation by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), 5 μL of 0.4 M GSH was added to 45 μL of the cytosolic proteins to derivatize unreacted [^{3}H]NEM and minimize any subsequent alkylation of disulfides that may occur when proteins are boiled in reducing electrophoresis buffer. Samples were analyzed for protein by the method of Lowry *et al.* [35] using bovine serum albumin as a standard. Proteins (30 μg/lane) were separated on a discontinuous 10% SDS–PAGE resolving gel with a 3% stacker [36] and stained with 0.025% Coomassie Blue R-250 in 25% methanol and 7% glacial acetic acid. After destaining, gels were impregnated with En^{3}Hance, dried, and exposed for 1–2 weeks to pre-flashed Kodak XAR-5 film at −70°.

Total protein thiols were quantified by reaction with 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) [37]. Cytosolic proteins (approximately 500 μg) were precipitated and washed with 1 N PCA to remove any non-covalently bound low molecular weight thiols. They were then resolubilized by heating at 60° for 10 min in 1.0 mL of 50 mM Tris buffer, pH 8.8, containing 0.1 mM EDTA, 2% SDS and a drop of antifoam A concentrate (Sigma). After the addition of 20 μL of a 20 mM DTNB chromogenic solution for 5 min, the optical density was read at 412 nm against a standard curve containing GSH as a reference thiol.

Assay of acetaminophen binding. The amount of acetaminophen bound was quantified *in vitro* by incubating cytosolic proteins with the reactive intermediate [^{3}H]NAPQI. Once bound to proteins, the NAPQI is recognized as acetaminophen immunochemically. The [^{3}H]NAPQI was synthesized chemically by addition of 200 μg of silver oxide to 25 μg of acetaminophen (containing 5 μCi of [^{3}H]acetaminophen) in 1.0 mL of HPLC grade acetonitrile [13]. The reaction was allowed to proceed at room temperature with constant stirring for 30 min. The resulting product was immediately passed through a 0.22 μM filtration disc to remove insoluble silver oxide. Serial dilutions from 1:1 to 1:4 (v/v) were made in additional acetonitrile and 25 μL of the resulting clear yellow solutions were mixed for 5 min with 5 mL of cytosolic proteins to yield final NAPQI concentrations equivalent to that

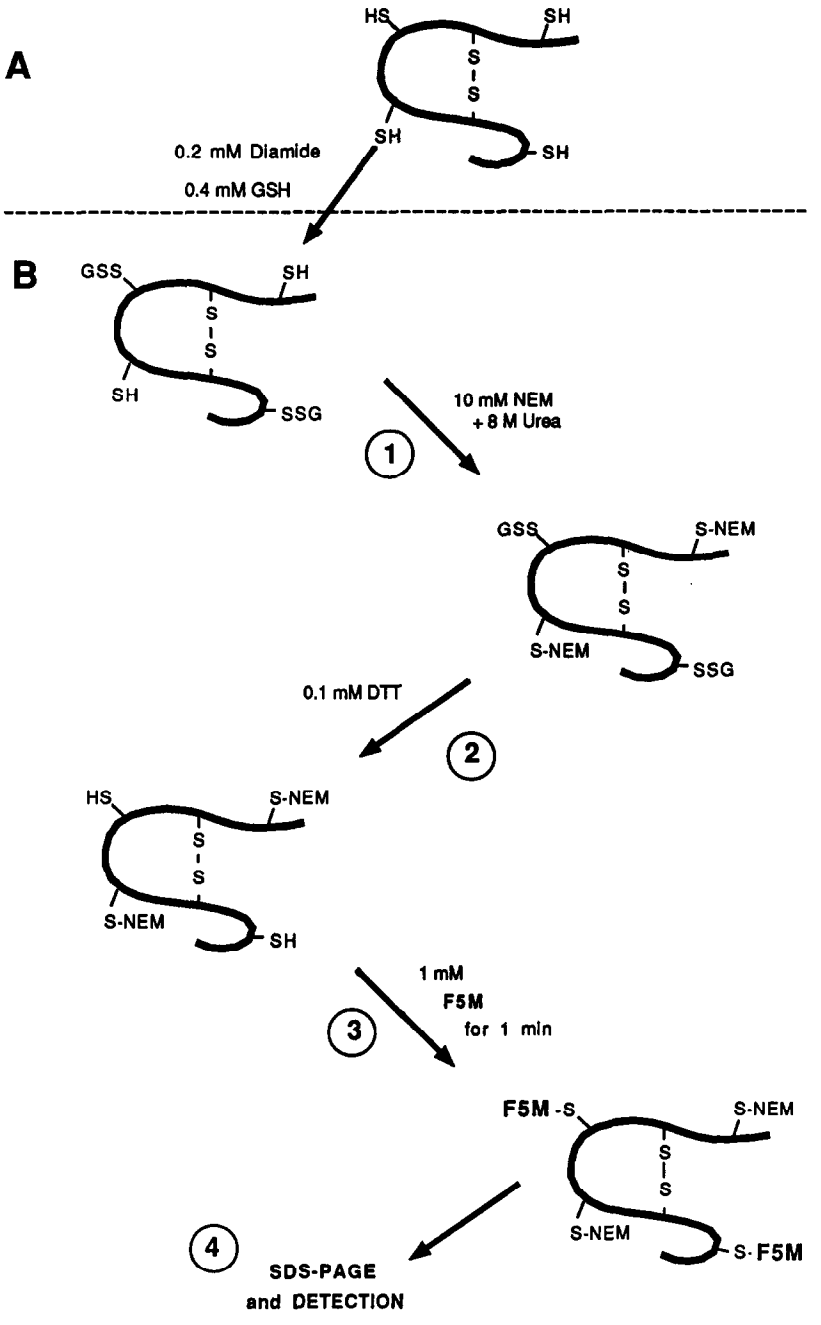

Fig. 1. Methodology to obtain and detect glutathiolated proteins. (A) To obtain "diamide-treated" samples containing glutathiolated-proteins, mouse liver cytosolic proteins were incubated for 10 min at room temperature with 0.4 mM GSH or [^{3}H]GSH (2.5 mCi/mmol) and up to 0.2 mM diamide. (B) To detect glutathiolated-proteins, samples were first treated with excess NEM (10 mM) and 8 M urea for 20 min to block accessible free protein thiol groups (Step 1). The urea was then removed by stepwise dialysis against diminishing urea concentrations (2 hr each with 6, 4, 2, 0.5 M urea) and finally against phosphate-buffered saline (PBS), pH 7.4. The proteins were then reduced with 0.1 mM DTT for 30 min at 37° to remove bound GSH (Step 2). The DTT was quickly removed by centrifugation through a mini-Sephadex G-25 column, and the protein thiols were exposed by removal of GSH, labeled with 1 mM fluorescein-5-maleimides for 1 min (Step 3). The reactions were stopped by the addition of excess GSH to alkylate unreacted maleimides. For quantification of mixed disulfide formation by liquid scintillation counting, 1 mM [^{3}H]NEM can be used to label the sites of glutathione binding. After separation by SDS–PAGE, the proteins were blotted to nitrocellulose membranes and probed for fluorescein-5-maleimide binding with anti-fluorescein antibodies, followed by ^{125}I-conjugated goat anti-rabbit IgG for detection by autoradiography (Step 4). Abbreviations: "SH" refers to available reactive thiols; "-SSG" refers to glutathione-protein mixed disulfides; "S-S" refers to inter- or intra-chain protein structural disulfides; and "F5M" refers to fluorescein-5-maleimide.

derived from the oxidation of 0.1 to 0.4 mM acetaminophen. The cytosolic proteins were precipitated with an equal volume of 2.0 N PCA and washed three times with 80% methanol containing 5 mM unlabeled acetaminophen and once with 1.0 N PCA. Protein pellets were solubilized in NCS, and the extent of acetaminophen binding was determined by liquid scintillation counting.

To detect acetaminophen-bound proteins, cytosolic proteins (30 μg) that had been incubated with NAPQI were separated by SDS–PAGE and electroblotted to 0.2 μm nitrocellulose membranes at 60 V for 6 hr. The membranes were blocked with 1% bovine serum albumin, incubated with affinity purified anti-acetaminophen antibodies (diluted 1:400), and then detected with ^{125}I-conjugated goat anti-rabbit IgG [17, 18]. Additional immunochemical studies were conducted with a polyclonal antibody (diluted 1:10,000) prepared against the purified 58-kDa acetaminophen binding protein [38]. The relative concentration of this protein target was determined by comparing the intensity of the immunoblot bands on X-ray film using an LKB Ultrascan XL laser densitometer.

Assay of glutathione-protein mixed disulfide formation (glutathiolation). To assay for proteins that form adducts with GSH upon exposure to diamide, cytosolic proteins were first passed over a Sephadex G-25 column (1.5 × 30 cm) equilibrated in PBS to remove endogenous GSH and other small molecules. The "diamide-treated" samples contained protein aliquots from the void volume which were then incubated for 10 min at room temperature with concentrations of diamide ranging from 0.05 to 0.5 mM in the presence of 0.4 mM GSH (Fig. 1A) [39]. However, concentrations greater than 0.2 mM diamide resulted in breakage of interdisulfide bonds. For "control" samples the livers were first homogenized in STM buffers containing 10 mM NEM to block exposed thiols, and the cytosolic proteins eluting in the Sephadex G-25 void volume were incubated with 0.4 mM GSH in the absence of diamide. The GSH concentration used was approximately equivalent to the concentration of NEM-reactive protein thiol groups in these aliquots. For quantification, 0.4 mM [^{3}H]GSH (2.5 mCi/mmol) was incubated with cytosolic proteins in the presence of up to 0.4 mM diamide and, after precipitation and washing three times with ice-cold 1 N PCA, the proteins were analyzed by liquid scintillation counting.

The method utilized to detect proteins that formed mixed disulfides is shown schematically in Fig. 1B. In step 1 all protein thiols not participating in either structural disulfides or protein mixed disulfides were blocked irreversibly by incubating the cytosolic proteins in 10 mM NEM containing 8 M urea. The unbound NEM and urea were then removed by dialysis. In step 2, the proteins were exposed to a concentration of dithiothreitol (DTT) (0.1 mM) that enabled release of protein-bound GSH without significantly breaking structural disulfide bonds. In step 3, the newly exposed thiol groups were labeled by a 1-min incubation with either radiolabeled NEM or fluorescein-5-maleimide. In step 4, proteins were either quantified by scintillation counting of the [^{3}H]NEM or separated by SDS–PAGE, and the maleimide-labeled proteins were detected by immunostaining with an anti-fluorescein primary antibody (1:100,000) and an ^{125}I-labeled secondary antibody.

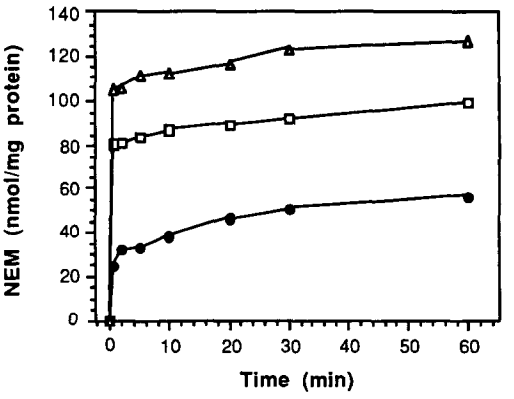

Fig. 2. Effect of time and urea on NEM binding in mouse liver cytosol. Cytosolic proteins (prepared by homogenizing mouse liver in 4 vol. of ice-cold PBS, pH 7.4, and centrifuging at 105,000 *g* for 60 min) were placed in either STM (●), STM containing 4 M urea (□), or STM containing 8 M (△) and reacted with 10 mM [^{3}H]NEM (0.1 mCi/mmol) for up to 60 min. At the indicated times, 250-μL aliquots were precipitated with an equal volume of ice-cold 2 N perchloric acid (PCA). The pellets were washed and [^{3}H]NEM binding was quantified by liquid scintillation counting as described in Materials and Methods. Data represent triplicate analyses.

Ability of acetaminophen binding to block diamide-induced glutathione binding to proteins. To determine the extent to which acetaminophen binding blocks GSH binding, cytosolic proteins were first reacted with 0.1 to 0.4 mM unlabeled NAPQI, dialyzed, and subsequently reacted with 0.4 mM [^{3}H]GSH and 0.2 mM diamide for 10 min at room temperature. Proteins were precipitated with 1.0 N PCA and then washed, and the extent of GSH-binding was determined by liquid scintillation counting.

RESULTS

Binding of NEM to reactive thiol groups. Under the denaturing conditions of the DTNB assay (pH 8.8 and 2% SDS), 120.7 ± 10.4 (SEM) nmol of total protein thiols were detected per mg of cytosolic protein. By contrast, at pH 7.4 under non-denaturing conditions, only about one-fourth (28.5 ± 0.8 nmol) of the thiols were titrated by [^{3}H]NEM after a 1-min incubation. The percentage of protein thiols that bind NEM could be enhanced by denaturing the proteins by the addition of 4 or 8 M urea to increase thiol accessibility. Incubation of cytosolic proteins with NEM in the presence of 8 M urea increased [^{3}H]NEM binding from 28.5 to 105 nmol/mg cytosolic protein (Fig. 2). The kinetics of NEM alkylation under both native and denaturing conditions appeared to be biphasic with an initial rapid binding phase followed by a slow continuous increase in binding. Although the extent of NEM binding in the first phase was dependent on the

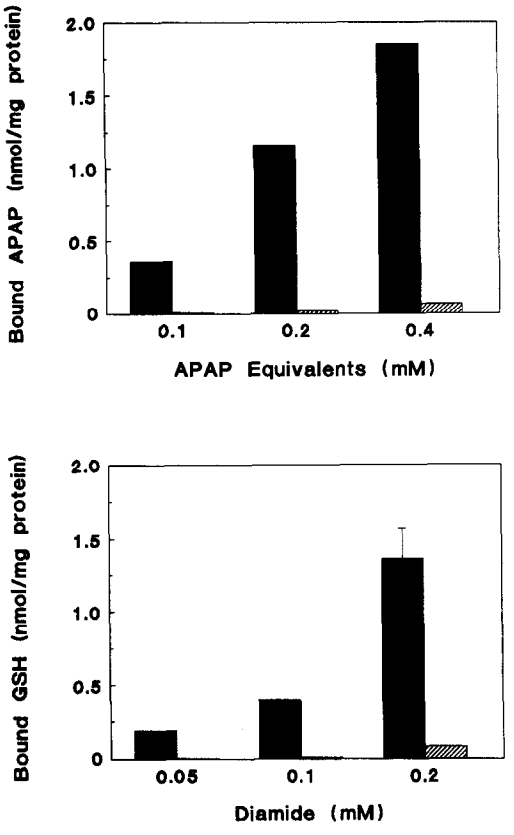

Fig. 3. Effect of NEM pretreatment on NAPQI and glutathione binding. Cytosolic proteins were incubated under non-denaturing conditions with (hatched bars) or without (solid bars) 10 mM unlabeled NEM for 1 min at 25°. Low molecular weight thiols and excess NEM were removed by passage through a Sephadex G-25 column equilibrated in PBS (1.5 × 30 cm). The proteins in the void volume were reacted with either chemically synthesized [^{3}H]NAPQI equivalent to 0.1, 0.2, or 0.4 mM acetaminophen (upper panel) or with 0.4 mM [^{3}H]GSH and 0.05, 0.1, 0.2 mM diamide (bottom panel). Proteins were precipitated with 1.0 N PCA and assayed for bound radioactivity as described in Materials and Methods. One experiment typical of three is shown. The SEM is indicated for the 0.2 mM diamide-treated data in the bottom panel.

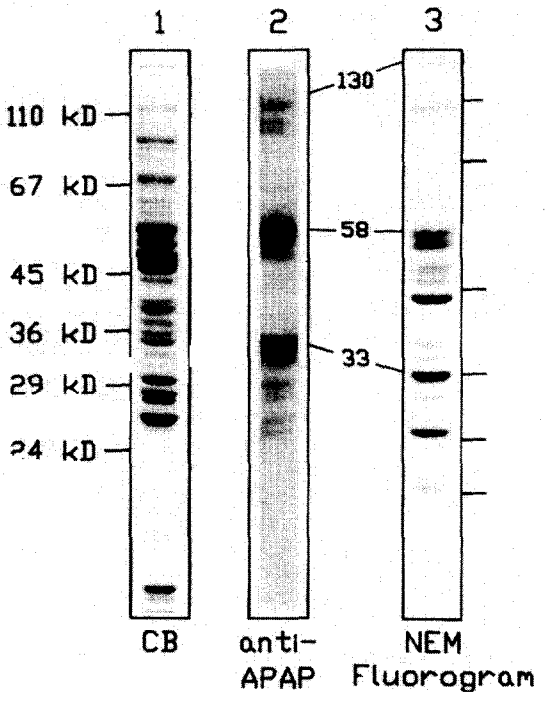

Fig. 4. Comparison of acetaminophen-binding protein adducts with [^{3}H]NEM-binding proteins. Cytosolic proteins (30 μg/lane) were resolved by 10% SDS–PAGE under reducing conditions and one lane was stained with Coomassie Blue R-250 (lane 1). The proteins in the other lanes were first reacted with either 10 mM [^{3}H]NEM (50 mCi/mmol) for 1 min or with chemically synthesized NAPQI equivalent to 0.1, 0.2, or 0.4 mM acetaminophen for 5 min. The [^{3}H]NEM-bound proteins (lane 3) were impregnated with fluorographic En^{3}Hance and exposed to X-ray film. The proteins were transferred to nitrocellulose and the acetaminophen-binding proteins were probed with an anti-acetaminophen antibody, and detected by autoradiography after the addition of ^{125}I-conjugated goat anti-rabbit IgG. The major acetaminophen-binding proteins and the corresponding electrophoretic migration of [^{3}H]-NEM-binding proteins in the fluorogram are illustrated by lines with molecular weights connecting lanes 2 and 3. The migration of the molecular weight standards is indicated.

presence of urea, the appearance of the slow reacting population of protein thiols was observed in both the presence and absence of urea (Fig. 2).

Ability of NEM pretreatment to block acetaminophen binding and diamide-mediated glutathiolation. The ability of NEM pretreatment to block protein thiols susceptible to either electrophilic attack by NAPQI or diamide-mediated glutathiolation *in vitro* was assessed by incubating cytosolic proteins with 10 mM NEM at pH 7.4 for 1 min (Fig. 3). Upon removal of excess NEM by Sephadex G-25 chromatography, subsequent incubation of these proteins with up to 0.4 mM [^{3}H]NAPQI demonstrated that NEM pretreatment blocked 97% of the binding of the electrophile (Fig. 3, top panel). When similarly treated proteins were incubated with [^{3}H]-GSH and up to 0.2 mM diamide, greater than 95% of GSH binding was also blocked (Fig. 3, bottom panel).

Additional studies examined the ability of acetaminophen binding to block diamide-induced GSH binding. At concentrations of NAPQI that resulted in covalent binding comparable to that observed after acetaminophen overdoses *in vivo* (about 1.8 nmol/mg cytosolic protein) [21, 40], subsequent GSH binding in the presence of 0.2 diamide was only inhibited about 12–18%.

Comparison of proteins which bind NEM and acetaminophen. To compare proteins that bind NAPQI *in vitro* to those that contain NEM-reactive thiols, an affinity-purified anti-acetaminophen antibody was used (Fig. 4). Few of the cytosolic proteins stained with Coomassie Blue (Fig. 4, lane 1) were arylated at concentrations of NAPQI that yielded covalent binding comparable to that observed *in vivo* (1.8 nmol/mg protein; Fig. 4, lane 2). Three of

the most prominent NAPQI-adducts (the 33, 58 and 130 kDa proteins) are similar in molecular weight to those detected immunochemically after acetaminophen toxicity *in vivo* [18, 21] and in culture [19, 34]. As anticipated from the data in Fig. 3, most of the arylated proteins, including the three major adducts, co-migrated with proteins that contained NEM-reactive thiols (Fig. 4, lane 3). As reported earlier [18], the 44 kDa acetaminophen binding protein adduct, that is observed *in vivo*, was not targeted by NAPQI *in vitro* (Fig. 4, lane 3).

Proteins that bind glutathione in the presence of diamide. To detect PSSG, a procedure was designed to reduce the mixed disulfide bonds with DTT and then label the newly exposed thiols with either radiolabeled NEM (for quantification) or fluorescein-5-maleimide (to visualize the specificity of protein glutathiolation) (Fig. 1B). The NEM-binding experiments described in Fig. 3 (lower panel) demonstrate that maleimides bind to the same pool of protein thiols that bind GSH after diamide treatment. To further validate the steps of this procedure it was necessary to: (a) demonstrate that NEM-accessible protein thiols not participating in disulfide bonds could be quantitatively and irreversibly blocked. This blocking is necessary to prevent free thiols from artifactually appearing as mixed disulfides in the final labeling step; and (b) demonstrate that relatively low concentrations of DTT could discriminate protein-mixed disulfide bonds from structural disulfide bonds.

To alkylate all protein thiols (Step 1, Fig. 1B), control and diamide-treated cytosolic proteins were passed through a Sephadex G-25 column and aliquots of the proteins from the void volume were reacted with 10 mM NEM in the presence of 8 M urea for 20 min. In the absence of urea or after the addition of only 4 M urea, the NEM pretreatment did not fully block the subsequent binding of [^{3}H]NEM (data not shown). This 8 M urea effect can probably be explained by the data in Fig. 2 whereby the presence of urea during the preincubation resulted in titration of a greater proportion of the total PSH. The inability to completely block [^{3}H]NEM binding in the absence of 8 M urea was not due to isotope impurities or tritium exchange since neither pre-absorption of the [^{3}H]NEM with control cytosol nor the use of [^{14}C]NEM increased the efficiency of blocking under such conditions.

Since the reduction of disulfide bonds by DTT is sensitive to the presence of protein denaturants [12], the urea had to be removed by stepwise dialysis. This step resulted in the precipitation of approximately 80% of the proteins in both control and diamide-treated samples. In addition, the removal of urea by dialysis also resulted in a decrease in the specific activity of the [^{3}H]GSH that is bound to proteins from 1.36 ± 0.21 nmol GSH/mg protein in control samples to 0.57 ± 0.09 nmol GSH/mg protein (mean ± SEM, N = 5) in the diamide-treated samples.

When the soluble proteins from the diamide-treated samples were reduced in the presence of increasing concentrations of DTT (Step 2, Fig 1B), 0.05 and 0.1 mM DTT resulted in an increase in [^{3}H]-NEM binding (Fig. 5). By contrast, when the soluble proteins from control samples were reduced in the presence of these concentrations of DTT, little additional [^{3}H]NEM binding above background was detected (Fig. 5). After treatment with 0.1 mM DTT, the diamide-treated proteins bound significantly more [^{3}H]NEM than did control proteins. At higher DTT concentrations, increased [^{3}H]NEM binding to control proteins was also observed.

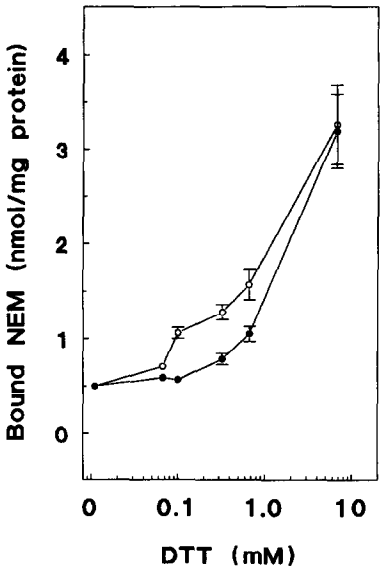

Fig. 5. Effect of DTT concentration on the reduction of protein disulfide bonds in liver cytosol. Mouse liver cytosolic proteins from control (●) and 0.2 mM diamide-glutathiolated (○) samples were prepared as described in Fig. 1. After dialysis to remove the urea (Step 1), the proteins were incubated with a range of DTT concentrations (0–5 mM) (step 2) and then labeled with 1 mM [^{3}H]NEM for 1 min and precipitated with an equal volume of ice-cold PCA prior to liquid scintillation counting. The DTT concentrations are plotted on a logarithmic scale. Data are the mean ± SEM of five independent experiments. Where no bars are shown, the SEM did not extend beyond the circles.

In parallel experiments, incubation of diamide-treated samples with 0.1 mM DTT resulted in the release of 80 ± 6% (SEM) of total protein-bound [^{3}H]GSH. In addition, in separate double-label experiments in which diamide-treated proteins were prelabeled with [^{3}H]GSH, reduced with 0.1 mM DTT and the newly exposed thiols labeled with [^{14}C]-NEM, the amount of newly bound [^{14}C]NEM was equivalent to the loss of protein bound [^{3}H]GSH (Fig. 6). When 5 mM DTT was used, this stoichiometric relationship was not observed and the amount of [^{14}C]NEM bound was at least 3-fold greater than the amount of [^{3}H]GSH removed. Collectively, these results suggest that the disulfides reduced with 0.1 mM DTT represent primarily PSSG, while at higher concentrations of DTT structural disulfide bonds are also disrupted.

To visually assess proteins that bind GSH during diamide treatment, the protein thiols exposed after reduction with 0.1 mM DTT were labeled with fluorescein-5-maleimide (Fig. 1B, step 3) and the

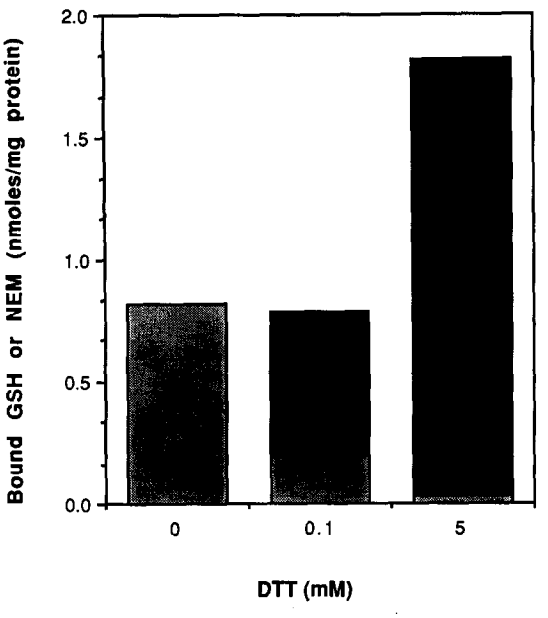

Fig. 6. Relationship between glutathione release and the appearance of NEM-reactive thiols. Cytosolic proteins were treated with 0.2 mM diamide in the presence of 0.4 mM [^{3}H]GSH. After incubation with or without 0.1 or 5 mM DTT, the proteins were labeled with [^{14}C]NEM for 1 min. The sample not reduced with DTT (0 mM DTT) depicts the amount of [^{3}H]GSH present on the protein after diamide treatment. Protein-bound [^{3}H]GSH is indicated by the light shading and [^{14}C]NEM binding is indicated by the dark hatched portions. Data are typical of several experiments utilizing different glutathione concentrations and were chosen because they were derived from the same experiment as that shown in Fig. 7.

fluorescein-bound cytosolic proteins were detected by immunostaining with an anti-fluorescein antibody (Fig. 1B, Step 4). Figure 7 represents electrophoretic separation of the cytosolic proteins remaining in solution after the removal of 8 M urea. Lane 1 depicts the proteins stained with Coomassie Blue. Lanes 2 through 5 represent anti-fluorescein-5-maleimide Western blots of the proteins from control and diamide-treated samples. Lane 2 contains control cytosolic proteins that were not reduced with DTT and, therefore, represent the background binding. Lane 3 contains diamide-treated proteins that were not reduced with DTT. This lane, therefore, depicts any spontaneous loss of bound glutathione. Lane 4 contains control proteins that were reduced with 0.1 mM DTT. Bands present in lane 4 that are absent in lane 2 therefore depict proteins which may contain endogenous mixed disulfides. Two such bands at 80 and 67 kDa are prominent. Lane 5 contains proteins from the diamide-treated samples that were reduced with DTT. The bands of 130, 58, 48, 38, 30, and 24 kDa, which are not prominent in the other lanes, indicate diamide-induced PSSG formation. The last lane (lane 6) contains NEM-reactive cytosolic proteins which were detected by fluorography after incubation with 10 mM [^{3}H]NEM (50 mCi/mmol). Although all the PSSG bands detected in lane 5 were also represented by co-migrating NEM-binding proteins in lane 6, the 30 and 58 kDa bands were most notable.

Evidence that the 58 kDa acetaminophen-binding protein is also glutathiolated after diamide treatment. A duplicate Western blot of control and diamide-treated proteins (corresponding to lanes 2 and 3 of Fig. 7) was probed with an antibody raised against the purified 58-kDa acetaminophen binding protein [38]. As expected, the bands labeled by this antibody (Fig. 8, lanes 2 and 3) had a migration identical to the 58-kDa protein detected with the anti-fluorescein antibody (lane 1) as well as the 58-kDa acetaminophen-binding protein detected with the anti-acetaminophen antibody (lane 4).

Evidence that the acetaminophen-binding protein can also become glutathiolated after treatment with diamide was provided by laser densitometric scans of the Western blots probed with the anti-58-kDa antibody. A comparison of control (Fig. 8, lane 2) and diamide-treated (Fig. 8, lane 3) lanes revealed that, per milligram of soluble cytosolic protein, about half of the 58-kDa acetaminophen-binding protein was lost during the removal of the urea in the diamide-treated sample. The loss of this protein in the diamide-treated sample paralleled the 58% decrease (from 1.36 to 0.57 nmol GSH/mg protein) in the specific radioactivity of [^{3}H]GSH bound to total cytosolic proteins. The proportional loss of the 58-kDa glutathione-binding proteins and the 58-kDa acetaminophen-binding protein supports the likelihood that the two 58-kDa proteins are the same.

DISCUSSION

To compare proteins that bind glutathione to those that bind acetaminophen, a new method for examining protein-mixed disulfides has been presented. This method detects very small quantities of thiol-binding proteins and does not require metabolic restrictions such as the inhibition of protein synthesis utilized in other methods [26]. It uses a maleimide derivative to both block protein thiols not participating in disulfide formation (detected with NEM) or to label protein thiols that have been exposed by reduction with DTT (detected with fluorescein-5-maleimide).

For the assay to exhibit minimal background binding, as many protein thiols as possible had to be initially blocked by reaction with NEM. This required the use of 8 M urea to expose buried protein thiols. Under more mild denaturing conditions, NEM was unable to block subsequent binding of either [^{3}H]NEM or fluorescein-5-maleimide. One explanation for the inability of NEM to block subsequent maleimide binding is that the presence of NEM causes a gradual denaturation of the protein, which continuously exposes new thiols. However, the observation that, even in the presence of 8 M urea, NEM binding continued to increase slowly for at least 60 min (Fig. 2) argues that a variable other than protein unfolding may be involved. Nevertheless, to minimize background binding, newly exposed thiols were labeled with maleimides using a short 1-min incubation.

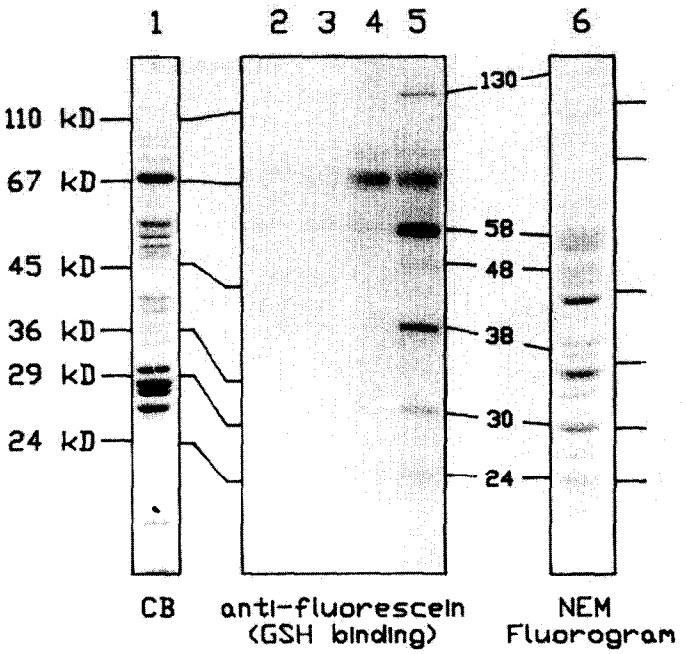

Fig. 7. Immunochemical detection of glutathiolated proteins. Diamide-treated proteins that remained soluble after urea removal were separated by SDS–PAGE and either stained with Coomassie Blue (lane 1) or Western blotted and immunostained (lanes 2–5). The Coomassie profiles from control and diamide-treated samples were not significantly different. Cytosolic proteins (6 μg/lane) from control (lanes 2 and 4) and 0.2 mM diamide-treated samples (lanes 3 and 5) were resolved on 10% SDS–PAGE and transferred to nitrocellulose membranes for probing with anti-fluorescein antibodies (diluted 1:100,000) followed by ^{125}I-conjugated goat anti-rabbit IgG. Lanes 2 (control) and 3 (diamide-treated) represent the binding of fluorescein-5-maleimide without prior reduction with DTT, while lanes 4 (control) and 5 (diamide-treated) represent fluorescein-5-maleimide binding after reduction with 0.1 mM DTT. These data were generated in the same experiment as shown in Fig. 6. For comparison, lane 6 represents cytosolic proteins that were reacted with 10 mM [^{3}H]NEM (50 mCi/mmol) for 1 min and then resolved by 10% SDS–PAGE under reducing conditions. The gels for lane 6 were impregnated with fluorographic En^{3}Hance and exposed to X-ray film. The major glutathione-binding proteins and the corresponding electrophoretic migration of [^{3}H]NEM-binding proteins in the fluorogram are illustrated by lines with molecular weights connecting lanes 5 and 6. The migration of the molecular weight standards is indicated by lines connecting lanes 1 and 2.

Since reduction of disulfide bonds by DTT is sensitive to the presence of protein denaturants [12], the 8 M urea which was used to help expose all protein thiols had to be removed prior to reduction with DTT. Removal of urea by sequential dialysis resulted in the loss of about 80% of both control and diamide-treated cytosolic proteins. This loss was not unexpected since many proteins do not fully return to their native configuration and precipitate out of solution following urea removal [37]. In addition, as indicated by the 58% drop in specific radioactivity of the protein-bound [^{3}H]GSH in the diamide-treated samples, protein mixed disulfides appeared to be more susceptible to this loss. This is likely due to a decrease in the stability of glutathiolated proteins [41]. Although it is not possible to establish whether some of these protein mixed disulfides are lost completely, a Coomassie Blue stain of the proteins that remain soluble is found to be representative of most cytosolic proteins (compare lanes 1 in Figs. 4 and 7). Despite the observed loss of protein, the high sensitivity of the fluorescein-immunoblot assay (3.4 pmol of GSH bound per the 6 μg of protein loaded per lane) readily permitted detection of the glutathiolated cytosolic proteins that did remain soluble.

The most critical step of the procedure required the establishment of optimal conditions that would break protein-mixed disulfide bonds with minimal rupture of structural disulfides. Hillson [42] had suggested that 0.1 mM DTT would be sufficiently discriminating. At this concentration of DTT, cytosolic proteins pretreated with diamide and GSH showed significantly more [^{3}H]NEM binding than did the protein fractions not treated with diamide (Fig. 5). Significantly, after treatment with 0.1 mM DTT, 80% of the protein-GSH mixed disulfide bonds were broken and the release of protein-bound [^{3}H]-GSH was found to be stoichiometric with the subsequent increase in [^{14}C]NEM binding (Fig. 6). These results provide three important pieces of information. First, they indicate that the brief 1-min incubation with maleimide to label exposed thiols was sufficient to bind these newly exposed thiols. Second, they suggest that the sites of GSH binding still react with maleimides after reduction with DTT.

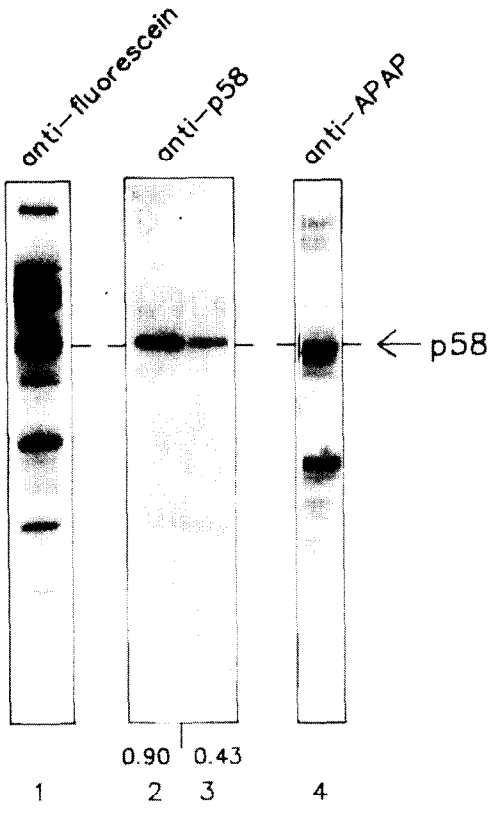

Fig. 8. Immunoblot analysis of p58. Lanes 2 and 3 represent a duplicate Western blot of control and diamide-glutathiolated proteins from Fig. 7 (lanes 2 and 3 of Fig. 7, respectively). The proteins were probed with an antibody directed against the 58-kDa major acetaminophen-binding protein [38]. The numbers under lanes 2 and 3 represent the relative absorbance units after laser densitometry scanning. For comparison, an anti-fluorescein blot of the major glutathiolated proteins (lane 1 which is identical to lane 5 of Fig. 7) and an anti-acetaminophen blot of the cytosolic proteins targeted by NAPQI *in vitro* (lane 4, which is identical to lane 2 of Fig. 4) are shown.

Third, they strongly suggest that the use of 0.1 mM DTT in this assay does not result in breakage of structural disulfide bonds that can subsequently bind maleimides and, therefore, permits adequate discrimination between protein mixed disulfides and structural disulfides even after NEM binding and the use of strong denaturing conditions.

The results of this study demonstrate that at least six cytosolic proteins with molecular weights of 130, 58, 48, 38, 30 and 24 kDa can become selectively glutathiolated upon mild diamide treatment. Similar selective PSSG formation has been reported in heart and liver cells exposed to diamide or *t*-butyl hydroperoxide [25–29]. A 30-kDa protein, recently identified as carbonic anhydrase III, readily undergoes S-thiolation in rat liver after diamide administration [29–30]. Furthermore, in a human lung carcinoma cell line (A427) exposed to hydrogen peroxide a protein of 38 kDa, identified to be glyceraldehyde-3-phosphate dehydrogenase, appears to undergo selective redox changes [31].

In this study GSH binding to cytosolic proteins was examined only *in vitro*. The amount of GSH bound when 0.2 mM diamide was used (1.36 ± 0.21 nmol/mg cytosolic protein) is within the range expected after exposure to an oxidative stress *in vivo* [43]. In addition, buffers containing NEM were used to avoid modification of protein thiols after cell lysis [11]. Although it is not yet possible to determine whether the same proteins will bind GSH in an intact cell system that is actively regulating its redox potential [44], the method can be adapted to more physiological conditions by treating cultured cells with diamide and then homogenizing the cells in the presence of NEM and urea to block free thiols.

Selective covalent binding of acetaminophen has also been demonstrated immunochemically [17–25]. This laboratory has provided evidence that acetaminophen binds to only a few proteins in mice [17–19, 21, 24] and in humans [22]. The amount of acetaminophen binding observed upon reaction with chemically synthesized NAPQI (0.2 to 1.8 nmol/mg protein) is also within the range reported after exposure to hepatotoxic doses of acetaminophen *in vivo* [21, 40] or in culture [19]. The arylation of the cytosolic 58-kDa protein, targeted by NAPQI *in vitro*, has been extensively correlated with the ensuing hepatotoxicity *in vivo* and in culture [17, 19, 21, 22, 24]. The lack of targeting of the 44-kDa acetaminophen binding protein upon the addition of NAPQI *in vitro* is not unexpected since this protein has been shown to contain few NEM-reactive thiols [18] and appears to become cytosolic only after its release from microsomal membranes that had become arylated by acetaminophen [23].

Under the *in vitro* conditions utilized in these studies, less than 2% of all protein thiols titratable with DTNB bound either acetaminophen or GSH. Although at pH 7.4, under non-denaturation conditions, NEM titrates only about 25% of the protein thiols, NEM pretreatment virtually eliminated the binding by both GSH and acetaminophen. These results suggest that both compounds bind to a pool of reactive protein thiols and that this pool is a subset of those thiols that bind NEM (Fig. 9). These data support the notion that the NEM-reactive thiols that bind acetaminophen and GSH are those that are most nucleophilic and accessible to soluble components. It also confirms that maleimides can be used to label sites that are susceptible to thiol–disulfide interchange by diamide.

Acetaminophen binding at levels comparable to those observed *in vivo* did not block more than about one-fifth of the glutathiolations induced by diamide (Fig. 9). The small extent of this blocking may indicate (1) that the sites of acetaminophen binding were not saturated at the concentrations of NAPQI utilized, or (2) that acetaminophen binding may alter the conformation of targeted proteins and expose new thiol sites for binding GSH. Alternatively, the lack of more extensive blocking may simply be related to the unique micro-environment surrounding each protein [45]. Since GSH and acetaminophen differ both structurally and electrostatically, differences in target binding should have been expected and were observed (compare Figs. 4 and 7).

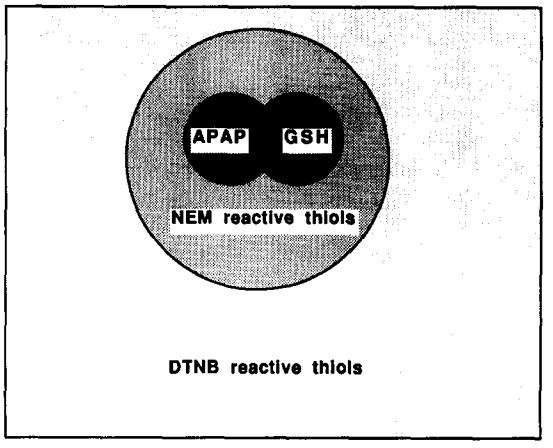

Fig. 9. Model illustrating the relationship between GSH and acetaminophen binding proteins and their thiol reactivity with NEM and DTNB. The model illustrates that NEM, under non-denaturing conditions, binds to about 25% of the protein thiols that react with DTNB, at pH 8.8 in the presence of 2% SDS. Acetaminophen and GSH bind to a subset of the NEM-reactive thiols and account for only about 2% of the protein thiols that bind DNTB. The overlapping inner circles (not drawn to scale) represent the relatively few proteins, such as the 58-kDa protein, that can bind both acetaminophen and GSH.

Despite differences in targeting, a comparison of proteins that bind GSH to those that bind acetaminophen reveals that at least one of the major cytosolic acetaminophen-binding proteins, the 58-kDa protein, was also glutathiolated upon diamide treatment. It is intriguing to speculate that reversible glutathiolation and irreversible acetaminophen arylation may be functionally related. If proteins that bind acetaminophen represent intracellular electrophile scavengers which protect the cell by decreasing the concentrations of reactive electrophiles such as NAPQI, then the oxidative-stress mediated glutathiolation of such proteins may be detrimental to the cell by decreasing the number of protective thiol sites on the protein. Alternatively, if such targeted proteins are essential to cell viability, their reversible glutathiolation under oxidative conditions may be a protective mechanism to safeguard these proteins from irreversible arylation by intermediates such as NAPQI. Identification and analysis of the function of proteins with such reactive thiols will enhance our understanding of the homeostatic role of PSSG and the significance of drug covalent binding in cellular toxicity.

Acknowledgements—This study was supported by grants from the University of Connecticut Research Foundation and by N.I.H. (GM-31460). R.B.B. was supported by an NIH postdoctoral fellowship in Environmental Toxicology (No. ES-07163). The authors would like to thank the staff of the University of Connecticut Biotechnology Center for their helpful technical assistance and Ms. Mary Bruno for her thoughtful discussions of this manuscript.

REFERENCES

1. Streeter AJ, Dahlin DC, Nelson SD and Baillie TA. The covalent binding of acetaminophen to protein. Evidence for cysteine residues as major sites of arylation *in vitro*. *Chem Biol Interact* **48**: 349–366, 1984.
2. Hoffmann KJ, Streeter AJ, Axworthy DB and Baillie TA. Identification of the major covalent adduct formed *in vitro* and *in vivo* between acetaminophen and mouse liver proteins. *Mol Pharmacol* **27**: 566–573, 1985.
3. DiMonte D, Ross D, Bellomo G, Eklow L and Orrenius S, Alterations in intracellular thiol homeostasis during the metabolism of menadione by isolated rat hepatocytes. *Arch Biochem Biophys* **235**: 334–342, 1984.
4. Thor H, Hartzell P, Svensson SA, Orrenius S, Mirabelli F, Marionin V and Bellomo G, On the role of thiol groups in the inhibition of liver microsomal Ca^{2+} sequestration by toxic agents. *Biochem Pharmacol* **34**: 3717–3723, 1985.
5. Brigelius R, Mixed-disulfides: Biological function and increases in oxidative stress. In: *Oxidative Stress* (Ed. Sies H), pp. 243–272. Academic Press, London, 1985.
6. Ziegler DM, Role of reversible oxidation-reduction of enzyme thiols-disulfides in metabolic regulation. *Annu Rev Biochem* **54**: 305–329, 1985.
7. Sies H, Brigelius R and Graf P, Hormones, glutathione status, and protein-S-thiolation. *Adv Enzyme Regul* **26**: 175–189, 1987.
8. Gilbert HF, Molecular and cellular aspects of thiol-disulfide exchange. In: *Advances in Enzymology and Related Areas of Molecular Biology* (Ed. Meister A), Vol. 63, pp. 69–172. John Wiley, New York, 1990.
9. Carraway KL and Shin BC, Specific modification, isolation, and partial characterization of an erythocyte membrane protein. *J Biol Chem* **247**: 2102–2108, 1972.
10. O'Donnell ME and Williams CH, Reactions of both active site thiols of reduced thioredoxin reductase with *N*-ethylmaleimide. *Biochemistry* **24**: 7617–7621, 1985.
11. Szajewski RP and Whitesides GM, Rate constants and equilibrium constants for thiol-disulfide interchange reactions involving oxidized glutathione. *J Am Chem Soc* **102**: 2011–2026, 1980.
12. Creighton TE, Pathways and energetics of protein disulfide formation. In: *Functions of Glutathione: Biochemical, Physiological, Toxicological, and Clinical Aspects* (Ed. Larsson A), pp. 205–213. Raven Press, New York, 1983.
13. Dahlin DC, Miwa GT, Lu AYH and Nelson SD, *N*-Acetyl-*p*-benzoquinone imine: A cytochrome P-450-mediated oxidation product of acetaminophen. *Proc Natl Acad Sci USA* **81**: 1327–1331, 1984.
14. Albano E, Rundgren M, Harvison PJ, Nelson SD and Moldeus P, Mechanisms of *N*-acetyl-*p*-benzoquinone imine cytotoxicity. *Mol Pharmacol* **28**: 306–311, 1985.
15. Rosen GM, Rauckman EJ, Ellington SP, Dahlin DC, Christie JL and Nelson SD, Reduction and glutathione conjugation reactions of *N*-acetyl-*p*-benzoquinone imine and two dimethylated analogues. *Mol Pharmacol* **25**: 151–157, 1984.
16. Moore M, Thor H, Moore G, Nelson S, Moldéus P and Orrenius S, The toxicity of acetaminophen and *N*-acetyl-*p*-benzoquinone imine in isolated hepatocytes is associated with thiol depletion and increased cytosolic Ca^{2+}. *J Biol Chem* **260**: 13035–13040, 1985.
17. Bartolone JB, Sparks K, Cohen SD and Khairallah EA, Immunochemical detection of acetaminophen bound liver proteins. *Biochem Pharmacol* **36**: 1193–1196, 1987.
18. Bartolone JB, Birge RB, Sparks K, Cohen SD and Khairallah EA, Immunochemical analysis of acetaminophen covalent binding to proteins. Partial

characterization of the major acetaminophen binding liver proteins. *Biochem Pharmacol* **37**: 4763–4774, 1988.

19. Birge RB, Bartolone JB, McCann D, Mangold J, Cohen SD and Khairallah EA, Selective protein arylation by acetaminophen and 2,6-dimethyl-acetaminophen in cultured hepatocytes from phenobarbital-induced and uninduced mice. Relationship to cytotoxicity. *Biochem Pharmacol* **38**: 4429–4438, 1989.
20. Bartolone JB, Beierschmitt WP, Birge RB, Emeigh Hart SG, Wyand S, Cohen SD and Khairallah EA, Selective acetaminophen metabolite binding to hepatic and extrahepatic proteins: An *in vivo* and *in vitro* analysis. *Toxicol Appl Pharmacol* **99**: 240–249, 1989.
21. Beierschmitt WP, Brady JT, Bartolone JB, Wyand DS, Khairallah EA and Cohen SD, Selective protein ayrlation and the age dependency of acetaminophen hepatotoxicity in mice. *Toxicol Appl Pharmacol* **98**: 517–529, 1989.
22. Birge RB, Bartolone JB, Emeigh Hart S, Nishanian EV, Tyson CA, Khairallah EA and Cohen SD, Acetaminophen hepatotoxicity: Correspondence of selective protein arylation in human and mouse liver *in vitro,* in culture, and *in vivo. Toxicol Appl Pharmacol* **105**: 472–482, 1990.
23. Birge RB, Bulera SJ, Bartolone JB, Ginsburg GL, Cohen SD and Khairallah EA, The arylation of microsomal membrane proteins by acetaminophen is associated with the release of a 44-kDa acetaminophen-binding mouse liver protein complex into the cytosol. *Toxicol Appl Pharmacol* **109**: 443–454, 1991.
24. Brady JT, Birge RB, Khairallah EA and Cohen SD, Post-treatment protection with piperonyl butoxide against acetaminophen hepatotoxicity is associated with changes in selective but not total covalent binding. In: *Biological Reactive Intermediates IV* (Eds. Witmer CM, Snyder RR, Jollow DJ, Kalf GF, Kocsis JJ and Sipes IG), pp. 685–688. Plenum Press, New York, 1990.
25. Pumford NR, Hinson JA, Benson RW and Roberts DW, Immunoblot analysis of protein containing 3-(cystein-*S*-yl)acetaminophen adducts in serum and subcellular liver fractions from acetaminophen-treated mice. *Toxicol Appl Pharmacol* **104**: 521–532, 1990.
26. Grimm LM, Collison MW, Fisher RA and Thomas JA, Protein mixed-disulfides in cardiac cells. S-Thiolation of soluble proteins in response to diamide. *Biochim Biophys Acta* **844**: 50–54, 1985.
27. Collison MW, Beidler D, Grimm LM and Thomas JA, A comparison of protein S-thiolation (protein mixed disulfide formation) in heart cells treated with *t*-butyl hydroperoxide or diamide. *Biochim Biophys Acta* **885**: 58–67, 1985.
28. Thomas JA and Beidler D, A thin-gel focusing method for quantitation of protein S-thiolation. *Anal Biochem* **157**: 32–38, 1986.
29. Rokutan K, Thomas JA and Sies H, Specific S-thiolation of a 30-kDa cytosolic protein from rat liver under oxidative stress. *Eur J Biochem* **179**: 233–239, 1989.
30. Chai Y-C, Jung C-H, Lii C-K, Ashraf SS, Hendrich, S, Wolf B, Sies H and Thomas JA, Identification of an abundant S-thiolated rat liver protein as carbonic anhydrase III; Characterization of S-thiolation and dethiolation reactions. *Arch Biochem Biophys* **284**: 270–278, 1991.
31. Brodie AE and Reed DJ, Cellular recovery of glyceraldehyde-3-phosphate dehydrogenase activity and thiol status after exposure to hydroperoxides. *Arch Biochem Biophys* **276**: 212–218, 1990.
32. Brigelius R, Lenzen R and Sies H, Increase in hepatic mixed disulfide and glutathione disulfide levels elicited by paraquat. *Biochem Pharmacol* **31**: 1637–1641, 1982.
33. Bellomo G, Mirabelli F, DiMonte D, Richelmi P, Thor H, Orrenius C and Orrenius S, Formation and reduction of glutathione-protein mixed disulfides during oxidative stress. A study with isolated hepatocytes and menadione (2-methyl-1,4-napthoquinone). *Biochem Pharmacol* **36**: 1313–1320, 1987.
34. Birge RB, Bartolone JB, Nishanian EV, Bruno MK, Mangold JB, Cohen SD and Khairallah EA, Dissociation of covalent binding from the oxidative effects of acetaminophen. Studies using dimethylated acetaminophen derivatives. *Biochem Pharmacol* **37**: 3383–3393, 1988.
35. Lowry OH, Rosebrough NJ, Farr AL and Randall RJ, Protein measurement with the Folin phenol reagent. *J Biol Chem* **193**: 265–275, 1951.
36. Laemmli UK, Cleavage of structrual proteins during the assembly of the head bacteriophage T4. *Nature* **227**: 680–685, 1970.
37. Sedlak J and Lindsay RH, Estimation of total, protein-bound, and non-protein sulfhydryl groups in tissues with Ellman's reagent. *Anal Biochem* **25**: 192–205, 1968.
38. Khairallah EA, Bartolone JB, Birge RB, Bruno MK, Nishanian EV and Cohen SD, Characterization of the major acetaminophen binding proteins. In: *Basic Science in Toxicology, Proceedings of the Fifth International Congress of Toxicology* (Eds. Volans GN, Sims J, Sullivan FM and Turner P), p. 137. Taylor & Francis, London, 1990.
39. Kosower NS, Kosower EM, Wertheim B and Correa W, Diamide, a new reagent for the intracellular oxidation of glutathione to the disulfide. *Biochem Biophys Res Commun* **37**: 593–596, 1969.
40. Jollow DJ, Mitchell JR, Potter WZ, Davis DC, Gillette JR and Brodie BB. Acetaminophen-induced hepatic necrosis. II. Role of covalent binding *in vivo. J Pharmacol Exp Ther* **187**: 195–202, 1973.
41. Liang JN and Pelletier MK, Destabilization of lens protein conformation by glutathione mixed-disulfides. *Exp Eye Res* **47**: 17–25, 1988.
42. Hillson DA, Resolution of thiol-containing proteins by sequential elution covalent chromotography. *J Biochem Biophys Methods* **4**: 101–111, 1981.
43. Brigelius R, Muchel C, Akerboom TPM and Sies H, Identification and quantitation of glutathione in hepatic protein mixed disulfides and its relationship to glutathione disulfide. *Biochem Pharmacol* **32**: 2529–2534, 1983.
44. Smith CV and Jaeschke HA, Effect of acetaminophen on hepatic content and biliary efflux of glutathione disulfide in mice. *Chem Biol Interact* **70**: 241–248, 1989.
45. Semenow-Garwood D, Model systems related to reactivity of protein sulfur functions. I. The effect of hydrophobic bulk on acid strengths of alkyl-substituted benzenethiols and on nucleophilicities of the benzenethiolate anions towards *N*-ethylmaleimide. *J Org Chem* **37**: 3797–3803, 1972.