Biochemical Pharmacology, Vol. 36, No. 8, pp. 1313–1320, 1987.
Printed in Great Britain.

0006-2952/87 $3.00 + 0.00

FORMATION AND REDUCTION OF GLUTATHIONE–PROTEIN MIXED DISULFIDES DURING OXIDATIVE STRESS

A STUDY WITH ISOLATED HEPATOCYTES AND MENADIONE (2-METHYL-1,4-NAPHTHOQUINONE)

G. BELLOMO,* F. MIRABELLI,* D. DIMONTE,* P. RICHELMI,* H. THOR,† C. ORRENIUS† and S. ORRENIUS†‡

*Dipartimento di Medicina Interna e Terapia Medica, Clinica Medica I[a], University of Pavia, 27100 Pavia, Italy, and †Department of Toxicology, Karolinska Institutet, S-104 01 Stockholm, Sweden

(*Received* 12 *August* 1986; *accepted* 31 *October* 1986)

Abstract—Incubation of isolated rat hepatocytes with menadione (2-methyl-1,4-naphthoquinone) resulted in a dose-dependent depletion of intracellular reduced glutathione (GSH), most of which was oxidized to glutathione disulfide (GSSG). Menadione metabolism was also associated with a dose- and time-dependent inhibition of glutathione reductase, impairing the regeneration of GSH from GSSG produced during menadione-induced oxidative stress. Inhibition of glutathione reductase by pretreatment of hepatocytes with 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) greatly potentiated both GSH depletion and GSSG formation during the metabolism of low concentrations of menadione.

Concomitant with GSH oxidation, mixed disulfides between glutathione and protein thiols were formed. The amount of mixed disulfides produced and the kinetics of their formation were dependent on both the intracellular GSH/GSSG ratio and the activity of glutathione reductase. The mixed disulfides were mainly recovered in the cytosolic fraction and, to a lesser extent, in the microsomal and mitochodrial fractions.

The removal of glutathione from protein mixed disulfides formed in hepatocytes exposed to oxidative stress was dependent on GSH and/or cysteine and appeared to occur predominantly via a thiol–disulfide exchange mechanism. However, incubation of the microsomal fraction from menadione-treated hepatocytes with purified glutathione reductase in the presence of NADPH also resulted in the reduction of a significant portion of the glutathione–protein mixed disulfides present in this fraction.

Our results suggest that the formation of glutathione–protein mixed disulfides occurs as a result of increased GSSG formation and inhibition of glutathione reductase activity during menadione metabolism in hepatocytes.

Intracellular reduced glutathione (GSH)‖ plays an important role in the detoxication of a variety of exogenous compounds [1–4]. GSH can act either as a nucleophile, forming conjugates with electrophilic drug and carcinogen metabolites, or as a reductant in the metabolism of hydroperoxides and free radicals [5, 6]. Hydroperoxide reduction is catalyzed by glutathione peroxidases present in both the cytosol and mitochondria of mammalian cells [7–10]. During this reaction, glutathione disulfide (GSSG) is formed, and the rate of GSSG formation has been proposed to represent an index of free-radical-induced oxidative stress [11].

Most of the GSSG formed in glutathione peroxidase-catalyzed reactions is subsequently reduced by glutathione reductase [12–15], and GSH is regenerated at the expense of NADPH. However, when the rate of GSSG formation exceeds that of its reduction, or when NADPH becomes rate-limiting for glutathione reductase, intracellular accumulation of GSSG occurs [16]. An efflux of GSSG follows its intracellular accumulation, and studies on the mechanism responsible for this GSSG extrusion have provided evidence for the existence of an ATP-dependent translocase in erythrocytes [17] and a GSSG-stimulated ATPase in the plasma membrane fraction of rat liver [18, 19].

If not reduced to GSH or extruded into the extracellular space, GSSG can interact with intracellular proteins to form glutathione–protein mixed disulfides. This may occur either spontaneously or enzymatically, and a specific thiol transferase has been isolated from rat liver cytosol [20]. Although the physiological significance of this reaction is not yet fully understood, early work indicated that protein-bound glutathione could serve as an "emergency" pool of glutathione which could be mobilized according to cell needs [21]. More recent studies have suggested that mixed disulfide formation represents a physiological mechanism for the modulation of enzyme activity and, thereby, of cell metabolism [22, 23]. Formation of glutathione–protein mixed disulfides has also been reported to occur during the development of toxic injury in lung [24] and perfused liver [25, 26] challenged with the redox active dipy-

‡ To whom correspondence should be addressed.

‖ Abbreviations used: GSH, glutathione, reduced form; GSSG, glutathione disulfide; DTT, dithiothreitol; BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea; menadione, 2-methyl-1,4-naphthoquinone; Hepes, 4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid; NEM, *N*-ethylmaleimide; PCMB, *p*-chloro mercuri-benzoic acid.

ridilium compound, paraquat, and organic hydroperoxides, respectively.

As a continuation of our previous studies of mechanisms of oxidative stress in intact cells [27–29], we now report on the formation of mixed disulfides between glutathione and protein thiols during the metabolism of menadione (2-methyl-1,4-naphthoquinone) by isolated rat hepatocytes. The amount of mixed disulfides produced, and the kinetics of their formation, were dependent on the intracellular GSH/GSSG ratio, the availability of reductants, GSH and cysteine, and the activity of glutathione reductase.

MATERIALS AND METHODS

Collagenase (grade II) and glutathione reductase were purchased from Boehringer (Mannheim, F.G.R.) and GSH, GSSG and cysteine from Sigma Chemical Co. (St. Louis, MO). 1,3-Bis(2-chloroethyl)-1-nitrosourea (BCNU) was from Bristol Laboratories (Stockholm, Sweden). Other reagents were obtained from local suppliers at the highest available grade of purity.

Male Sprague–Dawley rats (180–250 g), allowed free access to food and water, were used in all experiments. Hepatocytes were isolated by collagenase perfusion of the liver, as described in [30], and incubations were performed in rotating, round-bottomed flasks at 37° under an atmosphere of 95% O_2, 5% CO_2. Cell concentration was 10^6 cells/ml, and cell viability was assayed by the trypan blue exclusion test [30].

To inhibit glutathione reductase, hepatocytes were incubated for 30 min with BCNU (75 μM) in Krebs–Henseleit buffer, supplemented with 20 mM Hepes, pH 7.4, and amino acids at the concentrations indicated in [31], except for serine, glutamine, cysteine, and methionine which were present at 0.2 mM final concentration [15]. At the end of incubation, the cells were pelleted at 100 rpm, washed once, and resuspended in the same medium, lacking BCNU. The cells were then incubated for 2 hr to allow GSH (which had been partially depleted by the BCNU treatment) to be resynthesized. At the end of this second incubation, the cells were harvested, washed once, and finally reincubated in amino-acid-free Krebs–Heinseleit medium, supplemented with 20 mM Hepes, pH 7.4. This treatment resulted in 83–95% inhibition of glutathione reductase. However, in the experiments reported here, only hepatocytes whose glutathione reductase activity was inhibited by more than 90% were employed.

Glutathione reductase activity was measured by monitoring the oxidation of NADPH at 340–375 nm. The assay mixture contained 0.1 M potassium phosphate buffer, pH 7.6, 1 mM $MgCl_2$ 50 mM GSSG, 0.1 mM NADPH, 1% Triton X-100, and 5–7 $\times$ 10^4 hepatocytes, in a total volume of 1 ml.

For the quantitation of glutathione–protein mixed disulfides in intact cells, 10 ml of the incubation mixture was centrifuged to remove proteins which could have been released into the medium during the incubation procedure. The cell pellet was treated with 5% perchloric acid to precipitate proteins and the sample was allowed to stand on ice for 30 min. The pellet was washed twice in 5% perchloric acid and resuspended in 1.5 ml of 0.1 M Tris–HCl, pH 8. $NaBH_4$ (50 mg) was added together with 2 drops of *n*-octanol to prevent foam. The protein suspension was homogenized by treatment for 20 sec with a Polythron (set at 6), and then incubated at 40° for 30 min. After cooling on ice, 1 ml of 50% HPO_3 was added to remove $NaBH_4$ and to precipitate proteins, which were pelleted by centrifugation at 105,000 *g* for 15 min. The treatment with $NaBH_4$ caused the release of protein-bound glutathione into the medium. Subsequent quantitation of GSH was performed by the HPLC technique described by Reed *et al.* [32], using a Perkin–Elmer Series 4 liquid chromatograph equipped with an LCI-100 computer intergrator.

For isolation of subcellular fractions, 50 ml of a cell suspension were centrifuged at low speed, the pellet was resuspended in 5 vol. of 0.25 M sucrose, containing 10 mM Hepes, pH 7.4, and then homogenized with 20 passes in a tightly fitted Potter–Elvehjem homogenizer. The mitochondrial fraction was recovered as the 9000 *g* pellet, the microsomal fraction as the 105,000 *g* pellet, and the cytosolic fraction as the 105,000 *g* supernatant. Quantitation of glutathione–protein mixed disulfides present in subcellular fractions was performed as described for intact cells, using 2 mg protein/ml in 0.25 M sucrose, in a total volume of 2 ml, as starting material.

Protein concentration was measured according to Lowry *et al.* [33].

RESULTS

On the role of glutathione reductase in menadione-induced glutathione disulfide formation

Incubation of isolated rat hepatocytes with increasing concentrations of menadione resulted in a dose-dependent decrease in intracellular GSH level and a parallel increase in GSSG formation (Fig. 1). At low concentrations of menadione (<200 μM), the amount of GSSG produced, and the rate of its formation, were greatly enhanced by pretreatment of the cells with the glutathione reductase inhibitor, BCNU, indicating that the formation of GSSG was followed by its re-reduction to GSH by glutathione reductase (Fig. 1). However, at higher concentrations of menadione ($\geq$200 μM), there was a rapid and extensive accumulation of GSSG, suggesting that the rate of GSH oxidation now exceeded the rate of its re-reduction or, alternatively, that intracellular glutathione reductase activity was inhibited by high concentrations of menadione. The data shown in Fig. 2 indicate that the latter was in fact the case, in that there was a dose-dependent inhibition of glutathione reductase during the metabolism of menadione by isolated hepatocytes. Two observations suggest that oxidation (or arylation) of functional sulfhydryl group(s) may have been responsible for glutathione reductase inhibition by menadione. First, other thiol reagents such as PCMB and diamide also inhibited glutathione reductase activity and, second, the thiol reducing agent, dithiothreitol, was able to prevent menadione-induced enzyme inhibition (H. Thor and C. Orrenius, unpublished results).

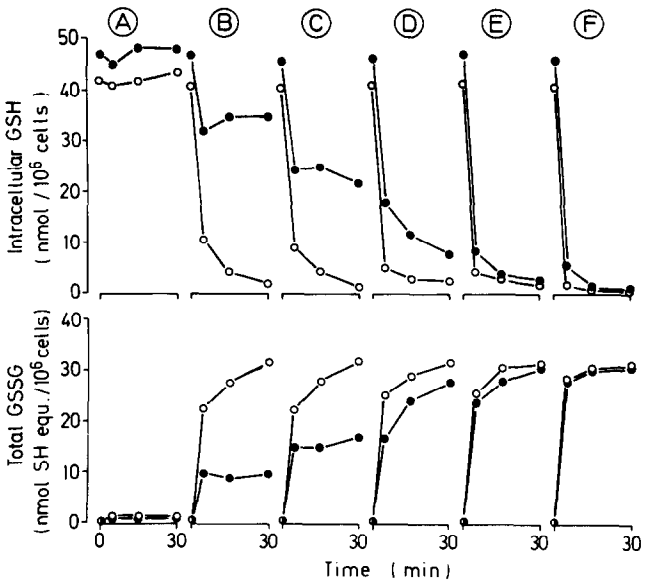

Fig. 1. Menadione-mediated oxidation of GSH in control and BCNU-treated hepatocytes. Hepatocytes were preincubated without (●) or with (○) BCNU in order to inhibit glutathione reductase, and then incubated without (A) or with 25 μM (B), 50 μM (C), 100 μM (D), 200 μM (E) and 400 μM (F) menadione. At the indicated times, samples were taken and processed for quantitation of intracellular GSH (upper panel) or total GSSG (intracellular + extracellular) (lower panel). One experiment typical of three is shown.

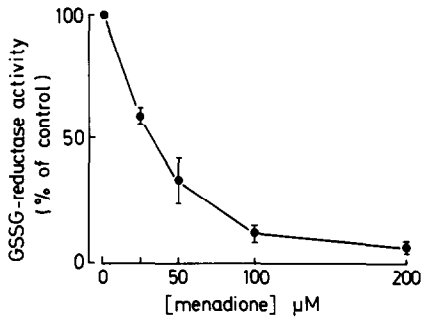

Fig. 2. Inhibition of glutathione reductase during the metabolism of menadione by isolated hepatocytes. Hepatocytes were treated for 20 min with the indicated concentrations of menadione, then, the activity of glutathione reductase was measured as described in Materials and Methods. Results are expressed as mean ± SD of three separate experiments.

Concomitant with GSH oxidation during menadione metabolism in hepatocytes, glutathione–protein mixed disulfides were formed in a dose-dependent manner (Fig. 3). The maximal amount of glutathione–protein mixed disulfides formed was 4.8 nmol/mg protein, corresponding to *ca.* 7.2 nmol/10^6 cells since, in our hands, 10^6 cells contain 1.5 ± 0.3 mg protein. It therefore appears that up to 15% of the total (reduced + oxidized) glutathione was bound to cell proteins as a result of menadione metabolism.

Similar to GSSG formation, mixed disulfide formation was potentiated by pretreatment of the cells with BCNU when low concentrations of menadione (<200 μM) were employed (Fig. 3, panels B, C and D). It is of interest to note that a maximal level of glutathione–protein mixed disulfides was reached already with the lowest concentration of menadione in BCNU-treated cells. This may be due to the limited reactivity of many protein sulfhydryl groups with respect to glutathione disulfide. Thus, in a previous study [34] we have found that in liver microsomes the maximal amount of mixed disulfides formed with glutathione disulfide (5 mM) was 6.2 nmol/mg protein whereas 18.9 nmol mixed disulfides/mg protein were formed with cystamine (5 mM).

The kinetics of glutathione–protein mixed disulfide formation in control cells treated with low doses of menadione was biphasic, showing rapid formation followed by a slow, but progressive, return to basal levels. In Fig. 4, the amount of glutathione–protein mixed disulfides formed during the metabolism of different concentrations of menadione has been plotted vs the intracellular GSH/GSSG ratio. Mathematical analysis of the distribution of the experimental values indicate a negative logarithmic correlation with a correlation coefficient (R) of −0.987 and a goodness of fit of 0.975, with N = 22. Thus, it appears that a critical GSH/GSSG ratio must be reached in order for glutathione–protein mixed disulfides to be formed.

Subcellular distribution of glutathione–protein mixed disulfides

The subcellular distribution of glutathione–protein mixed disulfides formed during the metabolism of menadione was analyzed by rapid fractionation of treated hepatocytes followed by quantitation of mixed disulfides in the different subcellular fractions. As illustrated in Fig. 5, most of the glutathione–

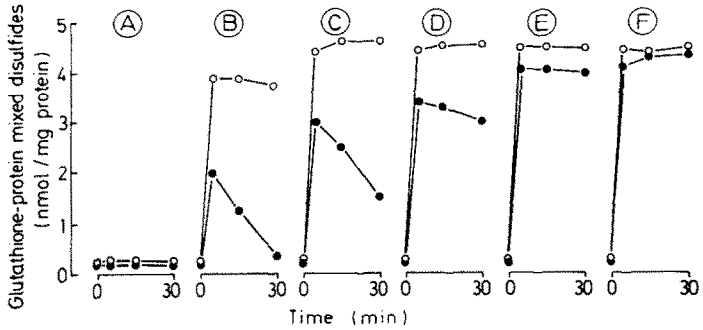

Fig. 3. Formation of glutathione–protein mixed disulfides during the metabolism of menadione by control and BCNU-treated hepatocytes. Hepatocytes were pretreated without (●) or with (○) BCNU in order to inhibit glutathione reductase. They were then incubated without (A) or with 25 μM (B), 50 μM (C), 100 μM (D), 200 μM (E) and 400 μM (F) menadione. At the indicated times, samples were taken and processed for quantitation of glutathione–protein mixed disulfides as described in Materials and Methods. One experiment typical of four is shown.

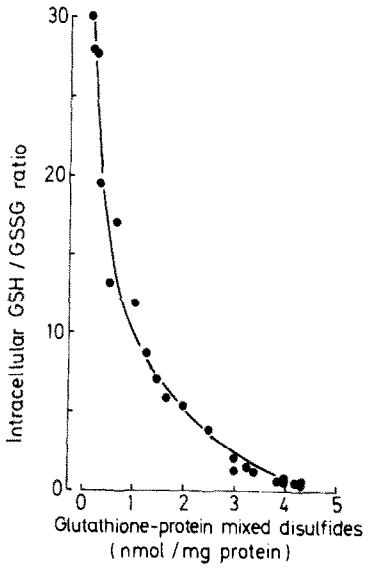

Fig. 4. Correlation between intracellular GSH/GSSG ratio and formation of glutathione–protein mixed disulfides during the metabolism of menadione by isolated hepatocytes. Hepatocytes were incubated with different concentrations of menadione as described in Figs 1 and 3, then samples were taken at different times for the simultaneous determination of intracellular GSH and GSSG and glutathione–protein mixed disulfides.

protein mixed disulfides were recovered in the cytosolic fraction, with lesser amounts in microsomes and mitochondria; the subcellular distribution of the mixed disulfides was unaffected by pretreatment of the hepatocytes with BCNU. The reason for the different distribution of glutathione–protein mixed disulfides in the subcellular fractions is not known, but it may reflect a greater availability of reactive protein thiols in the cytosolic proteins as compared to those present in the mitochondria and endoplasmic reticulum. In fact, in these organelles the percentage of sulfhydryl groups (expressed per mg protein) exposed to the cytosolic surface, and thereby to GSSG, is certainly low when compared to the cytosolic proteins.

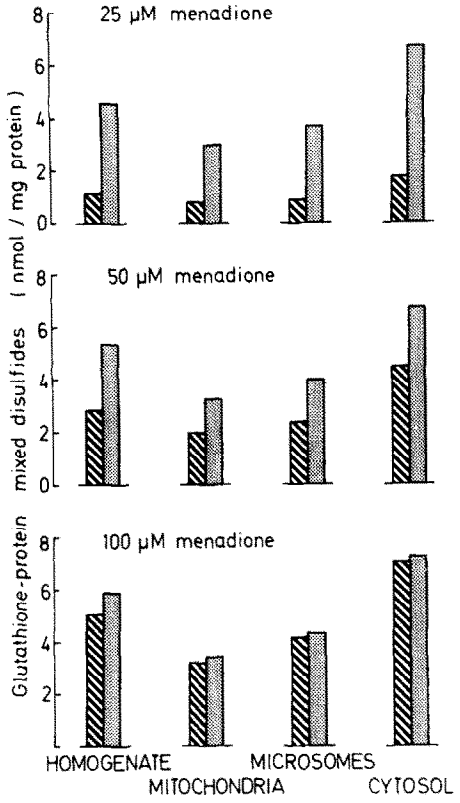

Fig. 5. Subcellular distribution of glutathione–protein mixed disulfides formed during the metabolism of menadione by control and BCNU-treated hepatocytes. Hepatocytes were preincubated without (▨) or with (□) BCNU in order to inhibit glutathione reductase. They were then incubated with the indicated concentrations of menadione. After 10 min, hepatocytes were washed in Krebs–Henseleit medium containing 1 mM NEM in order to prevent removal of any glutathione bound to proteins and to inhibit thiol–disulfide exchange. After standing for 10 min at room temperature, cells were processed as described in Materials and Methods for subcellular fractionation and quantitation of glutathione–protein mixed disulfides. One experiment typical of three is shown.

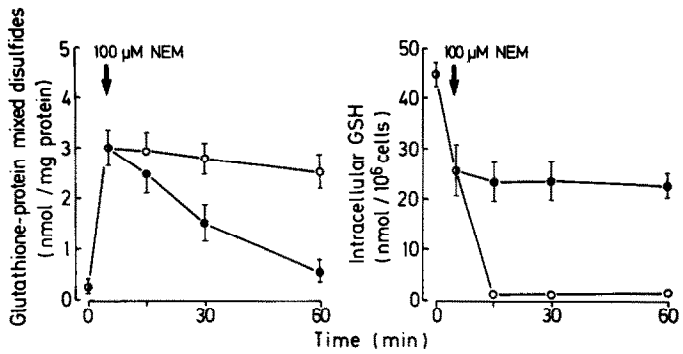

Fig. 6. Involvement of GSH in the removal of glutathione from protein mixed disulfides. Hepatocytes were incubated with 50 µM menadione. After 5 min, cell suspensions were divided into two aliquots, one of which (○) included 100 µM NEM in order to immediately deplete intracellular GSH. Samples were then taken at the indicated intervals for quantitation of intracellular GSH and glutathione–protein mixed disulfides. Results are expressed as mean ± SD of three separate experiments.

Since it is known that mitochondria contain a sequestered pool of GSH [35], the oxidation of this pool of glutathione during menadione metabolism could result in a compartment-specific formation of mixed disulfides. However, in our hands, the mitochondrial fraction isolated from hepatocytes contained *ca.* 5 nmol GSH/mg protein, four of which were recovered as GSSG after incubation of hepatocytes with 200 µM menadione for 10 min. Since more than 3 nmol of GSH were bound per mg of mitochondrial protein under these conditions, it seems likely that most of the glutathione recovered as mixed disulfides with mitochondrial proteins was cytosolic in origin.

On the role of GSH, cysteine, and glutathione reductase in the reduction of glutathione–protein mixed disulfides formed during the metabolism of menadione in hepatocytes

In order to investigate the role of GSH in the removal of the glutathione moiety from protein mixed disulfides, hepatocytes were treated with low concentrations of menadione (50 µM) to induce partial GSH oxidation and a transient formation of protein mixed disulfides. Then, after a short incubation (5 min), 100 µM *N*-ethylmaleimide (NEM) was added. As demonstrated in Fig. 6, this treatment resulted in a complete depletion of intracellular GSH and inhibition of the removal of glutathione from protein mixed disulfides. In a second set of experiments, hepatocytes were treated with a high dose of menadione (200 µM) which, in addition to causing a sustained formation of glutathione–protein mixed disulfides, induced an almost complete depletion of intracellular GSH. The cells were then washed and incubated in an amino-acid-containing medium to initiate GSH resynthesis. As demonstrated in Fig. 7(A), this treatment caused the reduction of glutathione–protein mixed disulfides which, however, could not be attributed to the resynthesis of GSH since it was not prevented by the simultaneous inclusion in the medium of the glutathione synthesis inhibitor, buthionine sulphoximine. Moreover, during the period of time in which the regeneration of protein thiols took place, the intracellular concentration of GSH did not change measurably (G. Bellomo and F. Mirabelli, unpublished results).

The possibility that cysteine, which was one of the amino acids present in the incubation medium, could act to remove glutathione from protein mixed disulfides was then investigated and found to be true, both in intact cells (Fig. 7A) and in microsomes isolated from menadione-treated cells (Fig. 7B). Moreover, addition of liver cytosol, which had been dialyzed to remove low molecular weight components, greatly enhanced the rate of cysteine-mediated removal of glutathione from protein mixed disulfides in microsomes isolated from menadione-treated cells (Table 1), suggesting that the reduction of protein mixed disulfides by cysteine was enzymatically mediated. In addition to cysteine, GSH was also effective in reducing glutathione–protein mixed disulfides and, again, this process was enhanced by addition of dialyzed cytosol (Table 1). Moreover, the demonstration that cytosol prepared from BCNU-treated cells was still active in accelerating both cysteine- and GSH-mediated reduction of mixed disulfides suggests that glutathione reductase was not involved in this process.

As mentioned above, NADPH-dependent glutathione reductase may also be involved in the reduction of glutathione–protein mixed disulfides in hepatocytes. This possibility was investigated using microsomes isolated from menadione-treated cells and subsequently incubated with purified glutathione reductase. As demonstrated in Fig. 8, neither glutathione reductase alone, nor NADPH, was able to decrease the amount of glutathione–protein mixed disulfides present in the microsomes. However, when glutathione reductase was added in combination with NADPH, *ca.* 40% of the glutathione bound to microsomal proteins was removed. The amount of GSH released was not increased by either prolongation of the incubation time, or by increasing the concentration of glutathione reductase and NADPH (not shown), suggesting that only a fraction of the glutathione bound to microsomal proteins was available for reduction by added glutathione reductase.

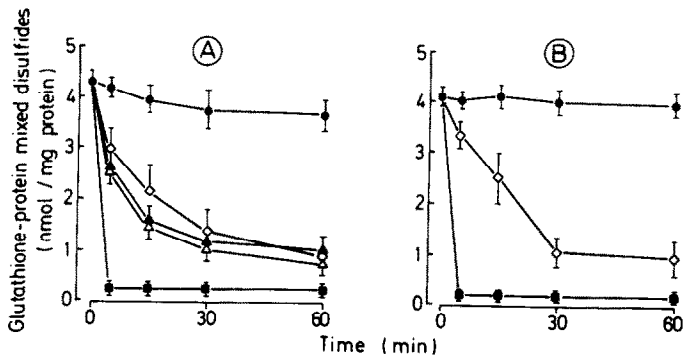

Fig. 7. Effects of dithiothreitol (DTT), amino acids and cysteine on the glutathione–protein mixed disulfides formed during the metabolism of menadione by isolated hepatocytes. In (A), hepatocytes were incubated for 30 min in the presence of 200 μM menadione, then washed and re-incubated in fresh Krebs–Henseleit medium alone (●) or supplemented with 2 mM DTT (■), 2 mM cysteine (◇) or a complete amino acid mixture (△, ▲) containing (▲), or lacking (△), 1 mM buthionine sulfoximine. In (B), microsomes were prepared from cells treated for 10 min with 200 μM menadione, then incubated (1 mg protein/ml) in a medium containing 0.1 M KCl and 10 mM Hepes, pH 7.4, alone (●) or supplemented with 2 mM DTT (■) or 2 mM cysteine (◇). Samples were taken at the indicated intervals for quantitation of glutathione–protein mixed disulfides. Results are expressed as mean ± SD of three separate experiments.

Table 1. Effects of cytosol from control and BCNU-treated cells on cysteine- and glutathione-mediated reduction of glutathione–protein mixed disulfides in microsomes isolated from menadione-treated cells

Additions	Glutathione–protein mixed disulfides (nmol/mg protein)
None	4.03 ± 0.38
Cysteine (2 mM)	3.31 ± 0.29
GSH (2 mM)	2.92 ± 0.31
Dialyzed cytosol (control cells) (100 μg protein/ml)	4.29 ± 0.35
Dialyzed cytosol (control cells) (100 μg protein/ml) + cysteine (2 mM)	2.11 ± 0.45
Dialyzed cytosol (control cells) (100 μg protein/ml) + GSH (2 mM)	1.88 ± 0.21
Dialyzed cytosol (BCNU cells) (100 μg protein/ml)	4.15 ± 0.38
Dialyzed cytosol (BCNU cells) (100 μg protein/ml) + cysteine (2 mM)	2.36 ± 0.14
Dialyzed cytosol (BCNU cells) (100 μg protein/ml) + GSH (2 mM)	2.09 ± 0.11

Microsomes were isolated from menadione-treated hepatocytes (200 μM menadione, 30 min) and then incubated in a medium containing 0.1 KCl and 10 mM Hepes, pH 7.4, at a final concentration of 1 mg protein/ml. Cysteine, GSH and/or dialyzed cytosol (from control or BCNU-treated cells) were added alone, or in combination, as indicated in the table. After 5 min at 37°, the reaction was stopped and the amount of glutathione–protein mixed disulfides was quantitated as described in Materials and Methods. Results are expressed as mean ± SD of three separate experiments.

DISCUSSION

In this paper we have presented data indicating that mixed disulfides are formed between glutathione and protein thiols during the metabolism of menadione by isolated hepatocytes. Several mechanisms appear to be involved in this process, including the oxidation of GSH to GSSG and the inhibition of glutathione reductase seen at high concentrations of menadione. Our findings are in agreement with previous reports by Brigelius *et al.* [25, 26], indicating that a strict correlation exists between the intracellular GSH/GSSG ratio and the amount of mixed disulfides formed between glutathione and protein thiols. A decrease in the GSH/GSSG ratio does not only provide the substrate (GSSG) required for protein S-thiolation, but it also appears that GSH depletion *per se* may exert an augmentative role in oxidative stress-induced protein mixed disulfide formation, since reduced glutathione, as demonstrated in this study, is an important factor in the removal of the glutathione moiety from protein

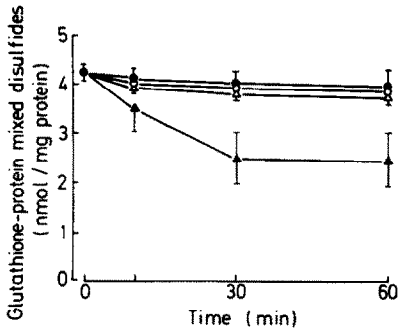

Fig. 8. Removal of glutathione from mixed disulfides by glutathione reductase. Microsomes, isolated from cells treated with 200 μM menadione for 10 min, were incubated (1 mg protein/ml) in a medium containing 0.1 M KCl, 1 mM $MgCl_2$ and 10 mM Hepes, pH 7.6, alone (●), or supplemented with 0.5 mM NADPH (○), 5 U/ml of glutathione reductase (△), or 0.5 mM NADPH + 5 U/ml of glutathione reductase (▲). Samples were taken at the indicated intervals for quantitation of glutathione–protein mixed disulfides. Results are expressed as mean ± SD of four separate experiments.

mixed disulfides. This hypothesis is also supported by previous reports on GSH-dependent diurnal variations in the content of glutathione–protein mixed disulfides in rat liver [36]. The finding that dithiothreitol efficiently removed all mixed disulfides formed during menadione metabolism provides further evidence for an important role of the intracellular redox status in controlling the formation of glutathione–protein mixed disulfides.

In addition to GSH, cysteine was able to reduce a substantial portion of the glutathione–protein mixed disulfides formed during the metabolism of menadione in hepatocytes. The mechanisms by which either GSH or cysteine can exert this effect has not been investigated in detail. However, it has previously been reported that diamide-induced glutathione–protein mixed disulfides in human erythrocytes are readily reduced by GSH via a thiol–disulfide exchange [37]. It seems very likely that cysteine exerts its effect through a similar mechanism according to the reaction:

$$\text{Prot-S-SG} + \text{cysteine} \rightleftharpoons \text{Prot-SH} + \text{GS–cysteine}$$

The detection of soluble cysteine–glutathione mixed disulfides when microsomes isolated from menadione-treated cells were incubated with cysteine (not shown) supports this hypothesis. Moreover, the finding that dialyzed rat liver cytosol greatly accelerated the rate of cysteine- and GSH-mediated mixed disulfide reduction in microsomes isolated from menadione-treated cells, suggests that this reaction is enzyme-mediated under physiological conditions, and probably involves the activity of thiol transferase [38].

The data reported in this study also provide evidence for a direct participation of glutathione reductase in reducing glutathione–protein mixed disulfides. However, the physiological significance of this reaction remains to be established. There is little doubt that the most important function of this enzyme in protecting against oxidative stress is related to the reduction of GSSG and thereby to the prevention of GSSG accumulation and mixed disulfide formation.

Although the metabolism of menadione is associated with the formation of glutathione–protein mixed disulfides, the role of this process in menadione-induced cytotoxicity is difficult to assess. A wide variety of enzyme and transport activities have been shown to be affected by the formation of mixed disulfides with functional protein sulfhydryls [38–43]. Among these are various Ca^{2+} transport systems and Ca^{2+}-stimulated ATPases [44–46] as well as Ca^{2+}-dependent protein phosphatases [47, 48]. It is therefore conceivable that mixed disulfide formation may contribute to the inhibition of Ca^{2+} transport and the perturbation of intracellular Ca^{2+} homeostasis occurring during oxidative stress in hepatocytes [49–51]. In a recent report we have shown that the formation of mixed disulfides between cystamine and a small pool of plasma membrane protein thiols results in an inhibition of Ca^{2+} extrusion and a sustained increase in cytosolic free Ca^{2+} concentration and subsequent cytotoxicity in hepatocytes [46]. However, it is not yet known whether a similar mechanism is effective in menadione-induced cell killing.

Acknowledgements—The expert technical assistance of Mr Paolo Crinó is gratefully acknowledged. This study was supported by grants from Consiglio Nazionale delle Richerche (Italy) and the Swedish Medical Research Council (project No. 03X-2471).

REFERENCES

1. L. F. Chasseaud, *Adv. Cancer Res.* **29**, 175 (1979).
2. H. Sies and A. Wendel (editors), *Functions of Glutathione in Liver and Kidney*. Springer, Berlin (1979).
3. D. J. Reed and P. W. Beatty, *Rev. Biochem. Toxic.* **2**, 213 (1980).
4. B. Ketterer, B. Coles and D. J. Meyer, *Envir. Hlth Perspect.* **49**, 59 (1983).
5. S. Orrenius, K. Ormstad, H. Thor and S. A. Jewell, *Fedn Proc. Fedn Am. Socs exp. Biol.* **42**, 3177 (1983).
6. D. J. Reed, *Biochem. Pharmac.* **35**, 7 (1986).
7. C. Little and P. J. O'Brien, *Biochem. biophys. Res. Commun.* **31**, 145 (1968).
8. N. J. Holmberg, *Expl Eye Res.* **7**, 570 (1968).
9. L. Flohé, W. A. Gunzler and H. H. Shock, *FEBS Lett.* **32**, 132 (1973).
10. A. Wendel, in *Enzymatic Basis of Detoxication*, Vol. 1 (Ed. W. B. Jakoby), p. 333. Academic Press, New York (1980).
11. B. H. Lauterberg, C. V. Smith, H. Huges and J. R. Mitchell, *J. clin. Invest.* **73**, 124 (1984).
12. R. E. Pinto and W. Bartley, *Biochem. biophys. Res. Commun.* **77**, 1257 (1969).
13. J. Lopez-Borea and C. Y. Lee, *Eur. J. Biochem.* **98**, 487 (1979).
14. D. P. Jones, L. Eklöw, H. Thor and S. Orrenius, *Archs Biochem. Biophys.* **210**, 505 (1981).
15. L. Eklöw, P. Moldéus and S. Orrenius, *Eur. J. Biochem.* **138**, 459 (1984).
16. L. Eklöw, P. Moldéus and S. Orrenius, in *Cytochrome P-450: Biochemistry, Biophysics and Environmental Implications* (Ed. E. Hietanen) p. 793. Elsevier–North Holland Biomedical, Amsterdam (1982).
17. S. Srivastava and E. Beutler, *J. biol. Chem.* **244**, 9 (1969).

18. P. Nicotera, M. Moore, G. Bellomo, F. Mirabelli and S. Orrenius, *J. biol. Chem.* **260**, 1999 (1985).
19. P. Nicotera, C. Baldi, S.-Å. Svensson, R. Larsson, G. Bellomo and S. Orrenius, *FEBS Lett.* **187**, 121 (1985).
20. R. Axelsson, S. Eriksson and B. Mannervik, *Biochemistry* **17**, 2978 (1978).
21. H. S. Kosower and E. H. Kosower, *Int. Rev. Cytol.* **54**, 109 (1978).
22. H. Gilbert, *J. biol. Chem.* **257**, 12086–12091 (1982).
23. R. Brigelius, in *Oxidative Stress* (Ed. H. Sies), p. 243. Academic Press, London (1985).
24. P. L. Keeling and L. L. Smith, *Biochem. Pharmac.* **31**, 3243 (1982).
25. R. Brigelius, R. Lenzen and H. Sies, *Biochem. Pharmac.* **31**, 1637 (1982).
26. R. Brigelius, C. Muckel, T. P. M. Ackerboom and H. Sies, *Biochem. Pharmac.* **32**, 2529 (1983).
27. H. Thor, M. T. Smith, P. Hartzell, G. Bellomo, S. Jewell and S. Orrenius, *J. biol. Chem.* **257**, 12419 (1982).
28. D. Di Monte, D. Ross, G. Bellomo, L. Eklöw and S. Orrenius, *Archs Biochem. Biophys.* **235**, 334 (1984).
29. D. Di Monte, G. Bellomo, H. Thor, P. Nicotera and S. Orrenius, *Archs Biochem. Biophys.* **235**, 343 (1984).
30. P. Moldéus, J. Högberg and S. Orrenius, *Meth. Enzym.* **51**, 60 (1978).
31. C. Waymouth and R. K. Jackson, *J. natl Cancer Inst.* **22**, 1003 (1959).
32. D. J. Reed, J. Babson, P. W. Beatty, A. E. Brodie and D. W. Potter, *Analyt Biochem.* **106**, 55 (1980).
33. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
34. G. Bellomo, P. Richelmi, F. Mirabelli, V. Marinoni and A. Abbagnano, in *Free Radicals in Liver Injury* (Eds. G. Poli, K. K. Cheeseman, M. V. Dianzani and T. F. Slater, p. 139. IRL Press, Oxford (1985).
35. O. W. Griffith and A. Meister, *Proc. natl Acad. Sci. U.S.A.* **82**, 4668 (1985).
36. J. Isaacs and F. Brinkley, *Biochim. biophys. Acta* **497**, 192 (1977).
37. C. W. M. Haest, D. Kamp and B. Deuticke, *Biochim. biophys. Acta* **557**, 363 (1979).
38. B. Mannervik and L. Axelsson, *Biochem. J.* **190**, 125 (1980).
39. R. Nesbakken and L. Eldjorn, *Biochem. J.* **87**, 526 (1963).
40. K. H. W. Lau and J. A. Thomas, *J. biol. Chem.* **258**, 2321 (1983).
41. A. Baba, E. Lee, T. Matsuda, T. Kihara and H. Iwaba, *Biochem. biophys. Res. Commun.* **85**, 1204 (1978).
42. T. Shimazu, S. Tokutake and M. Usami, *J. biol. Chem.* **253**, 7376 (1978).
43. F. H. Pettit, J. Humphreys and L. J. Reed, *Proc. natn. Acad. Sci. U.S.A.* **79**, 3945 (1982).
44. H. Thor, P. Hartzell, S.-Å. Svensson, S. Orrenius, F. Mirabelli, V. Moriani and G. Bellomo, *Biochem. Pharmac.* **34**, 3717 (1985).
45. J. P. Reeves, C. A. Bailey and C. C. Hale, *J. biol. Chem.* **261**, 4948 (1986).
46. P. Nicotera, P. Hartzell, C. Baldi, S.-Å. Svensson, G. Bellomo and S. Orrenius, *J. biol. Chem.* **261**, 14628 (1986).
47. M. M. King, *J. biol. Chem.* **261**, 4081 (1986).
48. E. A. Tallant and W. Y. Cheung, in *Calcium and Cell Function,* Vol. 6 (Ed. W. Y. Cheung), p. 71. Academic Press, New York (1986).
49. G. Bellomo, S. A. Jewell, H. Thor and S. Orrenius, *Proc. natn. Acad. Sci. U.S.A.* **79**, 6842 (1982).
50. G. Bellomo, S. A. Jewell and S. Orrenius, *J. biol. Chem.* **257**, 11558 (1982).
51. G. Bellomo, H. Thor and S. Orrenius, *FEBS Lett.* **168**, 38 (1984).