*Annu. Rev. Pharmacol. Toxicol. 1990. 30:603–31*




# GLUTATHIONE: Toxicological Implications

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KEY WORDS: protein thiols, mitochondrial glutathione, mechanisms of cytotoxicity, calcium homeostasis, cellular protective mechanisms

## INTRODUCTION

Glutathione as reduced glutathione (GSH) and as the disulfide (GSSG) is a unique peptide that continues to interest toxicologists—as reflected in the numerous symposia and reviews (1–6). This article undertakes to describe the role of mitochondrial glutathione in cellular response to toxic exposures that include loss of the homeostases of $Ca^{2+}$ and protein thiols. Because of the numerous reviews on the glutathione S-transferases (7–10), only the peroxidase activity of membrane-associated glutathione S-transferases is discussed.

Mammalian cells have evolved protective mechanisms to minimize injurious events that result from toxic chemicals and normal oxidative products of cellular metabolism. A major endogenous protective system is the glutathione redox cycle (2). Glutathione is present in high concentrations as GSH in most mammalian cells (generally in the millimolar range), with minor fractions being GSSG, mixed disulfides of GSH and other cellular thiols, and minor amounts of thioethers (1). GSH acts both as a nucleophilic "scavenger" of numerous compounds and their metabolites, via enzymatic and chemical mechanisms, converting electrophilic centers to thioether bonds, and as a substrate in the GSH peroxidase-mediated destruction of hydroperoxides. GSH depletion to about 20–30% of total glutathione levels can impair the cell's defense against the toxic actions of such compounds and may lead to cell injury and death (3, 11).



0362-1642/90/0415-0603$02.00

Endogenous oxidative stress is a consequence of aerobic metabolism, which, in eucaryotes, occurs mostly in the mitochondria. Reduction of oxygen in the respiratory chain involves the formation of toxic oxygen intermediates. About 2–5% of mitochondrial $O_2$ consumption generates hydrogen peroxide ($H_2O_2$) (12). Sies & Moss (13) concluded that mitochondrial generation of $H_2O_2$ under state 4 conditions (14–16) could require a turnover rate in the glutathione redox cycle of about 10% of the GSH per min. $H_2O_2$, if not reduced, can lead to the formation of the very reactive hydroxyl radical and cause the formation of lipid hydroperoxides that can damage membranes, nucleic acids, proteins, and alter their functions.

GSH can be depleted directly by conjugation with electrophiles and indirectly by the addition of inhibitors of GSH biosynthesis and regeneration (3, 17, 18). GSH is synthesized in vivo in the liver from γ-glutamylcysteine and glycine via GSH synthetase (EC 6.3.2.3), with the sulfur functionality of cysteine supplied by methionine via the cystathionine pathway (19). Inhibition of glutamylcysteine synthetase (EC 6.3.2.2), the cytosolic rate-limiting enzyme, by buthionine sulfoximine (BSO) diminishes the rate of GSH synthesis. GSH is maintained in a redox couple with GSSG within the cell and is regenerated by GSH reductase (EC 1.6.4.2), a cytosolic NADPH-dependent enzyme. Inhibition of this enzyme, and hence GSH regeneration, with 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) also depletes intracellular GSH (20). Alternatively, GSH content in isolated hepatocytes can be augmented by the addition of its sulfur-containing precursors, such as cysteine (or N-acetyl cysteine) or methionine, to maximize biosynthesis (21).

## MITOCHONDRIAL GSH

The presence of more than one pool of intracellular glutathione in liver was first suggested in 1952 (22) and was followed by the report that about 10% of the total thiol content of rat liver mitochondria was nonprotein-dialyzable thiol compounds such as glutathione (23). A major finding in 1973 was that glutathione in the mitochondrial matrix provided a reservoir of reducing equivalents capable of preventing the effects of oxidants on sensitive thiol groups (24). A complete glutathione redox system, which consists of GSH, glutathione reductase, glutathione peroxidase, and NADPH generated from NADH by transhydrogenation, exists in rat liver mitochondria (Figure 1). This system is capable of reduction of a wide range of diazenes and hydroperoxides (25). The effects of diamide [diazenedicarboxylic acid *bis* (N,N'-dimethylamide)] oxidation of glutathione and membrane thiol groups on mitochondrial functions include the perturbation of $Ca^{2+}$ homeostasis (26).

Mitochondrial GSH may be important in regulating inner membrane permeability by maintaining intramitochondrial thiols in the reduced state (27,

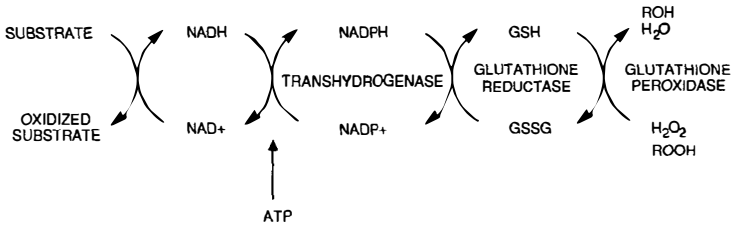


*Figure 1* Glutathione redox system present in mitochondria.



28). Certain proteins are highly sensitive to changes in the cellular thiol status, including the $Ca^{2+}$-dependent ATPases (29), which serve as membrane-bound $Ca^{2+}$ pumps to maintain cytoplasmic $Ca^{2+}$ at low levels. Interference of $Ca^{2+}$ homeostasis and increased levels of cytoplasmic-free $Ca^{2+}$ are believed to participate in cell injury (30) that, if not reversed, will eventually lead to cell death. Homeostases of $Ca^{2+}$ and of thiols in the mitochondria are believed to be closely linked either directly (28) or through the pyridine nucleotides (31); an imbalance in one could affect the status of the other, and diminish cell viability.

Because mitochondria have no catalase (32), they rely solely on GSH peroxidase to detoxify hydroperoxides (12). Glutathione peroxidase uses the reducing equivalents of GSH, the most abundant cellular nonprotein thiol, 10–15% of which is located in the mitochondria (33–35). A protective role for mitochondrial GSH in cytotoxicity was first proposed by Meredith & Reed (35, 36). They showed that the onset of cell injury in isolated rat hepatocytes by ethacrynic acid correlated with the depletion of mitochondrial GSH, whereas the cytosolic pool could be depleted without affecting cell viability. Previously, several reports (37–41) had demonstrated that cytotoxicity, as measured by lipid peroxidation, liver necrosis, and loss of intracellular enzymes in vivo and in vitro, occurred only if the intracellular concentrations of GSH fell below 10–15% of the initial value, which is the amount associated with mitochondria.

*t*-BuOOH treatment is accompanied by a decrease in intracellular GSH and NADPH concentrations and a release of GSSG from the perfused rat liver (42) and isolated rat hepatocytes (43). The rate of GSSG release is believed to be proportional to the activity of GSH peroxidase (44). GSSG is formed in isolated mitochondria during oxidative stress induced by *t*-BuOOH treatment, but, despite a drastic decrease in the mitochondrial GSH/GSSG ratio, no release of GSSG into the medium occurs (45). This was true when GSSG levels were quickly reduced back to GSH, also during a sustained perturbation of the GSH/GSSG ratio, or even when GSSG levels were greater than 50% of total mitochondrial glutathione for 60 min. The possibility that exported GSSG may have been reduced in the media was discounted by the finding that

no GSH equivalents were missing inside the mitochondria after glutathione levels recovered to those of nontreated mitochondria. These results suggest that a transport system for GSSG efflux is absent in liver mitochondria and that all GSSG formed inside mitochondria must be reduced in situ.

These results indicate that the redox status of mitochondrial GSH may influence intramitochondrial protein thiol groups and the integrity of mitochondrial membranes (27). The lack of ability to export GSSG indicates that mitochondria may be more susceptible to protein thiol oxidation than the rest of the cell and may explain why the loss of mitochondrial GSH, rather than cytosolic GSH, is critical in some types of cell injury (35, 46).

GSH is continuously lost at a slow rate from coupled mitochondria, which suggests an apparent efflux pathway for GSH in rat liver mitochondria. Earlier studies had shown an outward diffusion of GSH from mitochondria, with 25–56% of the endogenous level gone after 10 min incubation at 30 °C (33). As coupled mitochondria are impermeable to protons, and 2 mM GSH in the medium did not affect the rate of GSH release, a simple diffusion down a concentration gradient cannot explain the loss of GSH from isolated mitochondria (45).

A small but significant amount of mitochondrial GSH (approximately 10%) could not be depleted by raising the concentration of *t*-BuOOH during exposure of mitochondria (45). These data are in agreement with the results of Jocelyn & Cronshaw (47), who used chlorodinitrobenzene to deplete GSH and suggested that GSH might be sequestered within the mitochondrial matrix. A major new finding is the ability to oxidize selectively mitochondrial glutathione in cultured rat adrenal cells by certain polycyclic aromatic hydrocarbons such as the potent carcinogen 7,12-dimethylbenz[a]anthracene and its liver metabolite, 7-hydroxymethyl-12-methylbenz[a]anthracene but not the unmethylated benzo[a]pyrene or benzo[a]anthracene. The induced cytotoxicity has been suggested to result from uncoupling mitochondrial cytochrome P-450s to generate sufficient oxidative stress from reactive oxygen species to cause mitochondrial but not cytosolic GSSG formation (48). Chronic ethanol feeding to rats impairs the reaccumulation of mitochondrial GSH as the cytosolic pool increases and suppresses the mitochondrial GSH/cytosolic GSH ratio (49).

Benzoyl peroxide (BPO) induced mitochondrial damage that is characterized by inhibition of respiration and induction of rapid large-amplitude swelling. The mechanism of this damage appears to be dissociated from radical production and lipid peroxidation (50). Although BPO is not readily metabolized by mitochondrial glutathione peroxidase, the reaction between BPO and GSH is rapid and results in the formation of GSSG. Kennedy et al (50) have concluded that mitochondrial thiol groups are oxidized during BPO-induced swelling. A similar mechanism has been implicated in

mitochondrial swelling induced by chlorinated napthoquinones (51, 52). Of importance is the view that as BPO inhibits mitochondrial electron transport at coupling site II and inhibits respiration, radical production occurs but is scavenged in intact respiring mitochondria.

The results indicate that the mechanism of BPO-induced swelling is different from that of hydroperoxide-induced swelling, which is thought to be dependent on calcium ion cycling (53–55). Another important aspect of hydroperoxide induced-swelling is the consumption of reducing equivalents via GSH along with hydroperoxide as a substrate for glutathione peroxidase and the competition for the energy required to maintain both cellular ionic balance and thiol/disulfide redox status.

Two mechanisms of mitochondrial damage by exposure of rat hepatocytes in culture to *t*-BuOOH have been based on the effects of an antioxidant, N,N'-diphenylenediamine (DPPD). Mitochondrial membrane potential ($\Delta\varphi$) is lost as the principal effect at high hydroperoxide concentration (56). Interestingly, lipid peroxidation appears of major importance in the other mechanism, which involves hydroperoxide at low concentrations, with both mechanisms being dependent on a cellular source of ferric iron (56). Iron induces dose-dependent oxidative damage to isolated rat liver mitochondria by a proposed interaction with mitochondrial hydrogen peroxide. The damage is greater in state 4 than in state 3 respiration, which suggests that superoxide anion radical dismutation and radical damage occur in the mitochondrial matrix (57).

Masaki et al have demonstrated that the toxic consequences of a loss of mitochondrial energy due to exposure of cultured hepatocytes to various toxic agents can be prevented by a reduction of pH in the culture medium which causes intracellular acidosis (58). These workers have concluded that, with potassium cyanide treatment, cell killing [loss of lactate dehydrogenase (LDH)] is better correlated with a loss of mitochondrial energy and collapse of the $\Delta\varphi$ than depletion of ATP. Monensin, a proton/sodium ion ionophore, potentiated the toxicity of cyanide and carbonyl cyanide m-chlorophenylhydrazone (CCCP) (58). The toxicity of valinomycin was reduced, and swelling could be more of a dominant factor in the loss of mitochondrial membrane potential with this agent than with cyanide and CCCP (58).

Increased permeability of mitochondrial inner membrane induced by calcium ion plus inorganic phosphate, diamide, or hydroperoxides appears to involve oxygen radicals through some mechanism that is very responsive to the free radical scavenger, butylhydroxytoluene (59).

Acetaminophen hepatotoxicity is characterized by GSH depletion, covalent binding of reactive intermediate(s), and increased expiration of ethane and pentane. Acetaminophen failed to increase the hepatic content or biliary

efflux of GSSG in rats and mice (60). Smith & Jaeschke have concluded that oxidant mechanisms are not participating in hepatic injury in vivo by acetaminophen. However, a marked change in production or disposition of fatty acid hydroperoxides appears to be occurring. If the ability of glutathione to participate in fatty acid hydroperoxide metabolism is localized in the mitochondria of the heptocytes, any GSSG formed would not be expected to efflux (45). Failure of the mitochondria to efflux GSSG could account for the lack in vivo of GSSG efflux into bile.

Mitochondria energized by substrate oxidation show enhanced reaction of protein thiols with N-ethylmaleimide (NEM) and other maleimide derivatives (61, 62). Unmasking of certain thiol groups, which are positioned in a hydrophobic environment, is the reason given for the increase in the thiol reactivity (62). Loss of mitochondrial inner membrane selectivity for the transport of solutes after alkylation of these hydrophobic thiol groups results in the formation of holes 3 nm in size in the inner membrane (62, 63). These effects appear closely related to calcium homeostasis.



## CALCIUM AND MITOCHONDRIAL GSH

Over a decade ago, the release of calcium ion from liver mitochondria was found to be stimulated by oxidants that oxidized pyridine nucleotides (31). De-energized rat liver mitochondria undergo extensive swelling when incubated in the presence of calcium ion and diamide (an oxidant of GSSG) selected protein thiols, and pyridine nucleotides (64). The swelling could be inhibited by ruthenium red (RR), and the inhibition completely removed by the calcium ion ionophore, ionomycin. Vercesi et al have concluded that calcium ion cycling is not necessary and that calcium ion could bind to internal sites of the inner membrane to cause mitochondrial damage (64).

Calcium ion release from intact mitochondria can be induced by a variety of chemically different prooxidants (65). An important concept is the relationship to the oxidation of pyridine nucleotides leading to the release of calcium ion from rat liver mitochondria. According to Richter & Frei (65), the oxidized intramitochondrial pyridine nucleotides are hydrolyzed at the bond between the nicotinamide and ADP-ribose moiety and, in turn, calcium ion release is regulated by protein ADP-ribosylation. The observed transient rise in the level of intramitochondrial protein ADP-ribosylation during prooxidant-induced calcium ion release is followed by the return to the starting level of ribosylation when calcium ion release diminishes (Figure 2) (65).

The lipid soluble ionophore, A23187, has been used to study the role of divalent cations, principally $Ca^{2+}$, in various biological systems (66-69). At physiological concentrations of extracellular $Ca^{2+}$, Reed & Lardy (66) observed that A23187 induced the uptake and accumulation of $Ca^{2+}$ in

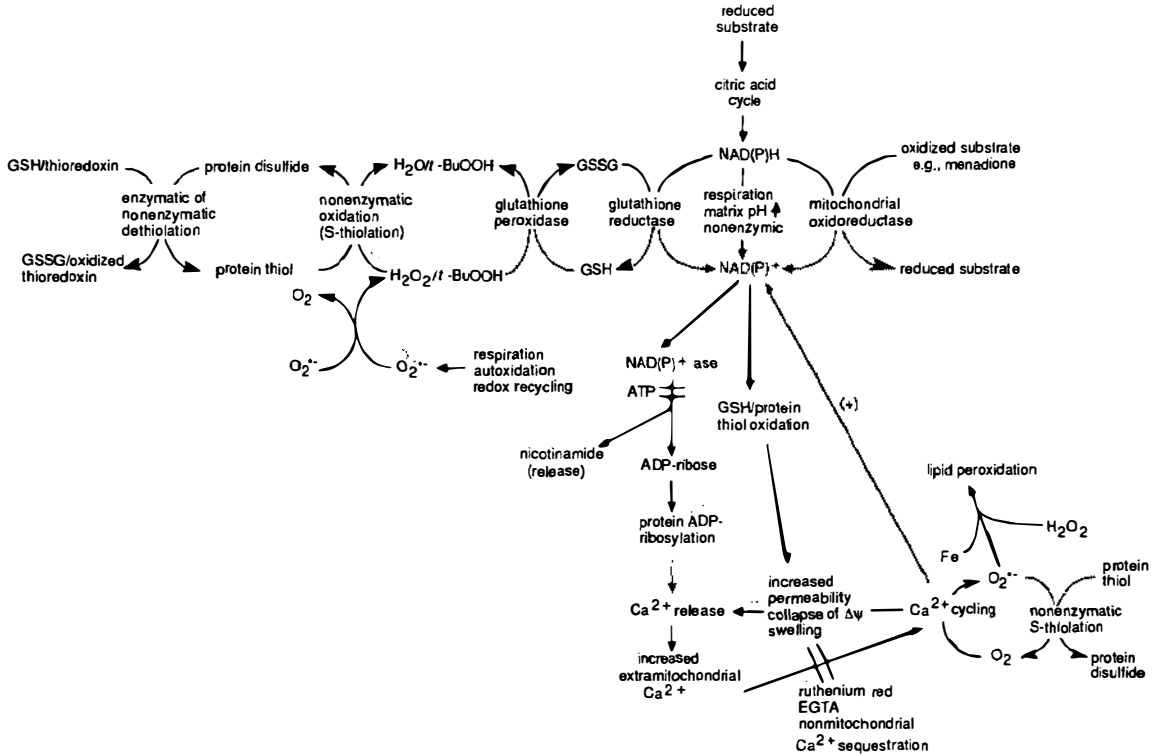


*Figure 2* Relationship of the homeostasis of calcium and thiols to the mitochondrial electron transport system. Endogenous oxidative stress is depicted as resulting from superoxide anion radical and hydrogen peroxide. Exogenous oxidative stress is typified with *t*-BuOOH and menadione. Since protein thiol oxidation and reduction mechanisms are not well understood, homeostasis of protein thiols is related to several factors as discussed by Gilbert and Houk et al (168, 169). This scheme is adapted from (65).

exchange for proton release from erythrocytes. They also found that A23187 inhibited mitochondrial ATPase by releasing endogenous $Mg^{2+}$, while uncoupling oxidative phosphorylation by inducing an energy-dissipating cyclic flux of $Ca^{2+}$ (66). At low extracellular $Ca^{2+}$ concentrations, the ionophore induced the efflux of intracellular $Ca^{2+}$ from isolated rat hepatocytes (67). By controlling the $Ca^{2+}$ concentration in the extracellular medium in the presence of A23187, we were able to examine some of the biochemical consequences of altered $Ca^{2+}$ homeostasis in isolated hepatocytes. The expression of chemically induced toxicity strongly correlated with mitochondrial GSH depletion, which was preceded by the loss of cytosolic GSH, whereas the loss of protein-SH appeared to occur after the loss of nonprotein thiols (70).

Ionophore-induced perturbation of cellular $Ca^{2+}$ homeostasis suggests that increased levels of cytoplasmic-free $Ca^{2+}$ precede the onset of cell death (30). However, whether this increase results from the influx of $Ca^{2+}$ from the environment (71, 72) or from the release of stored intracellular $Ca^{2+}$ to the cytosol (73, 74) has been extensively debated. Major decreases in the levels of cytosolic and mitochondrial GSH, with mitochondrial GSSG being increased, were caused by influx of extracellular $Ca^{2+}$ induced by A23187 as well as the disturbance of intracellular $Ca^{2+}$ homeostasis induced by A23187 in the absence of extracellular $Ca^{2+}$. When mitochondrial GSH was decreased to about one half of control level, a good correlation with the loss of cell viability was observed. The duration of depletion of mitochondrial GSH appears to be important. For example, the level of mitochondrial GSH present at 4 and 5 hr is approximately the same for three $Ca^{2+}$ concentrations; the toxicity correlates very well with the length of time mitochondrial GSH levels are 50% or less, compared to controls (46). Ionophore-induced cell injury and loss of mitochondrial GSH were both decreased by lowering the dose of A23187 or by lowering the $Ca^{2+}$ concentration in the medium.

Further evidence for the importance of mitochondrial GSH in cell injury is provided by the observations that agents which afforded protection against the loss of mitochondrial GSH also reduced cell injury. The antioxidants DPPD and vitamin E-succinate reduced the loss of mitochondrial GSH during calcium ionophore treatment and lessened the loss of cell viability (46). Since ionophore-induced $Ca^{2+}$ loss or accumulation in the hepatocytes was not affected by the presence of vitamin E, the antioxidants did not appear to prevent the incorporation of A23187 into cellular membranes. Vitamin E-succinate prevents both chemical-induced and nonchemical-induced injury to hepatocytes and appears to aid in the maintenance of nonprotein (75) and protein thiols (70, 76–78). The ability of DPPD to similarly prevent losses of protein-SH groups and mitochondrial GSH, concomitant with maintenance of cell viability, suggests that the thiol-sparing action is not specific to vitamin E, but is related to its antioxidant properties.

The absence of extracellular $Ca^{2+}$ causes oxidative stress that can be prevented by various agents (79–81). Particularly striking was the effect of the antioxidants on the loss of mitochondrial GSH and cell viability in the absence of extracellular $Ca^{2+}$. Under these conditions, loss of mitochondrial GSH was completely prevented by the presence of DPPD or vitamin E-succinate, which correlated well with their total prevention of cell injury. Thus, the ionophore-induced toxicity in the absence of extracellular $Ca^{2+}$ appears related to the consequences of alterations in intracellular $Ca^{2+}$ homeostasis that were preventable by both an increased antioxidant level in the cell and even by the ionophore during the first 1–2 hrs of incubation.

In the presence of extracellular $Ca^{2+}$, antioxidants could not totally prevent A23187-induced toxicity in hepatocytes (46). This suggests a different mechanism of toxicity from that which operates in the absence of extracellular $Ca^{2+}$. Further support for this contention was lent by the differential action of RR on A23187-induced toxicity. In the absence of $Ca^{2+}$, RR partially prevented cell injury, whereas it potentiated toxicity in the presence of $Ca^{2+}$ (46). This indicates that $Ca^{2+}$ cycling played a role in cell injury when the flux of $Ca^{2+}$ was directed out of the cell due to $Ca^{2+}$ omission from the medium. In the presence of $Ca^{2+}$, increased toxicity might be due to increased levels of cytoplasmic-free $Ca^{2+}$, as occurred when reuptake of $Ca^{2+}$ into the mitochondria was inhibited by RR or allowed free exchange by A23187. It has been shown that metabolism of toxic concentrations of *t*-BuOOH decreases the intracellular level of free $Ca^{2+}$ (82). Addition of substrates that provide reducing equivalents to the pyridine nucleotides prevented release of $Ca^{2+}$ from the mitochondria (82).

GSH and pyridine nucleotide oxidation in mitochondria have been shown to increase the permeability of the inner membrane to $Ca^{2+}$ (26, 31, 83) through the oxidation of protein thiol groups (84). This suggests that oxidative stress or severe GSH depletion may affect the redox status in the mitochondria enough to alter intracellular $Ca^{2+}$ homeostasis, which is believed to be an early step in cell injury (85, 86). During severe oxidative stress, high levels of GSSG could have deleterious effects on cell integrity and metabolic processes. Several investigators have shown that GSSG is actively released from cells undergoing an oxidative challenge (43, 44, 87, 88). Eklöw et al (43) demonstrated a rapid and extensive release of GSSG from hepatocytes treated with *t*-BuOOH, with only a transient increase in GSSG inside the cells before efflux. The GSSG efflux was preceded by a marked decrease in the cellular $NADPH/NADP^+$ redox level, supporting an earlier contention (89) that the GSSG efflux pathway is an important process for the cell to avoid highly oxidative states. Although it has been assumed to exist (90), no evidence exists for the operation of this pathway in the mitochondria. Therefore, the evidence that GSSG fails to efflux from mitochondria during oxidative stress

(45) indicates that hepatocyte mitochondria risk loss of thiol-dependent functions.

## OXIDATIVE STRESS BY OMISSION OF EXTRACELLULAR CALCIUM

The preceding section indicated the occurrence of oxidative stress associated with changes in calcium and thiol homeostases. However, the role of oxidative stress, and lipid peroxidation in particular, in chemically induced cell injury remains controversial (81). In the absence of extracellular $Ca^{2+}$ alone, significant formation of malondialdehyde (MDA) occurs prior to LDH leakage. Also, a marked loss of GSH and vitamin E can be noted immediately, thereby indicating an acute oxidative stress in these cells (79, 80). Incubation under an atmosphere of 100% $O_2$, rather than 95%, resulted in nearly 100% LDH leakage by 3 hr, whereas significant $K^+$ leakage, but little LDH leakage, occurred under an atmosphere of 95% air (i.e. 21% $O_2$) (91). Although it is difficult to demonstrate conclusively that oxidative stress is responsible for cell death, the available evidence suggests that, in the absence of extracellular $Ca^{2+}$, alterations in cellular function and/or integrity induced by oxidative stress are responsible for the resultant cell injury. Therefore, aspects of this hepatocyte model system will be discussed in some detail.

Neither the nature of the event(s) initiated by the lack of $Ca^{2+}$ which apparently induces the enhanced production of active oxygen species, nor the exact underlying cause of loss of cell viability is presently understood. The importance of the mitochondria in both cellular $Ca^{2+}$ homeostasis (92) and the maintenance of cellular integrity (35) has led to speculation that the absence of extracellular $Ca^{2+}$ may markedly affect normal mitochondrial function. The contention that mitochondria are affected by the omission of extracellular $Ca^{2+}$ is supported by the rapid and nearly complete loss of mitochondrial GSH (79, 80).

These data suggest that mitochondrial $Ca^{2+}$ cycling may be involved in oxidant stress and cell injury (Figure 3). Such a cycling process can be inhibited with RR, which even at concentrations as low as 10 $\mu$M, is very protective against oxidative stress as determined by MDA production, GSH oxidation, protein thiol oxidation, and vitamin E loss (79, 80). The protective effects of RR exhibited no dose response in the range of 10–100 $\mu$M, in agreement with the demonstration that RR binds to the uniport and inhibits $Ca^{2+}$ passage in a noncompetitive manner (93).

$Ca^{2+}$ cycling, resulting from efflux through the antiport, and subsequent uptake through the uniport can also decrease $\Delta\varphi$ (94), and thus it is clear that $\Delta\varphi$ and mitochondrial $Ca^{2+}$ homeostasis are intimately related. Therefore, it was of interest to determine whether the lack of extracellular $Ca^{2+}$ affected

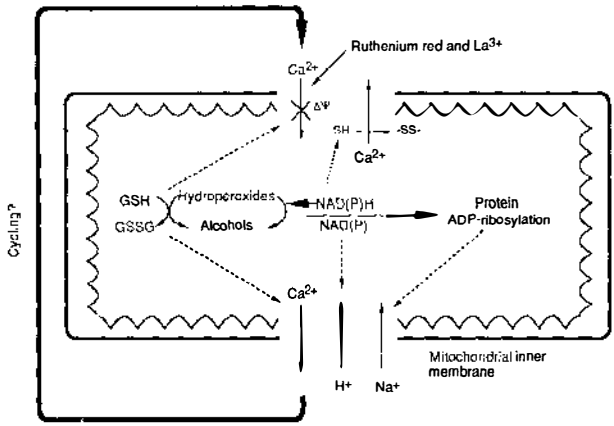


*Figure 3* Diagrammatic representation of mitochondrial $Ca^{2+}$ cycling. Depicted are the mechanisms by which the redox state of mitochondria [NAD(P)H/NAD(P)] can affect the uptake and efflux of $Ca^{2+}$ via processes involving GSH and ADP-ribosylation. Also indicated is the possibility of $Ca^{2+}$ cycling and the site at which ruthenium red and $La^{3+}$ block $Ca^{2+}$ uptake.

$\Delta\varphi$. It was observed that the omission of $Ca^{2+}$ resulted in a marked drop of $\Delta\varphi$ in viable hepatocytes (viability defined as the ability to centrifuge through a layer of dibutyl phthalate). The loss of [$^3$H]methyltriphenylphosphonium ([$^3$H]TPMP) indicated that $\Delta\varphi$ declined to less than 50% of initial values prior to significant LDH leakage (80). RR and EGTA both effectively prevented the loss of $\Delta\varphi$ (80).

It has been speculated that $Ca^{2+}$ leaves the mitochondria in response to lowered cytosolic free-$Ca^{2+}$ levels (95), which may occur in the absence of extracellular $Ca^{2+}$. Although the role of the mitochondria in regulation of cytosolic free $Ca^{2+}$ has been questioned (96), this pathway for efflux cannot be discounted. The finding that vitamin E and desferrioxamine also prevented the decrease in $\Delta\varphi$ induced by the omission of extracellular $Ca^{2+}$ (80) suggests several other alternative means by which mitochondrial $Ca^{2+}$ efflux may be induced. First, it has been reported that the oxidation of pyridine nucleotides promotes the efflux of $Ca^{2+}$ through the antiport (31, 97). The absence of $Ca^{2+}$ promotes GSH oxidation which stimulates NADH and NADPH oxidation via the activity of the mitochondrial $NAD(P)^+$ transhydrogenase and the GSH redox cycle (31, 98). Subsequent $Ca^{2+}$ cycling is a potentially deleterious energy-consuming process leading to a depression of $\Delta\varphi$ as demonstrated by Bellomo et al (54). Recent work also has provided evidence that lipid peroxidation of mitochondria induces the activation of a specific $Ca^{2+}$-release route without damaging mitochondrial integrity and that resultant $Ca^{2+}$ cycling leads to a loss of $\Delta\varphi$ (99).

Alternatively, initial oxidative damage to the mitochondria may lead to

alterations in permeability properties of the inner membrane. Thus, it is conceivable that, in the absence of $Ca^{2+}$, subtle changes such as oxidation of critical membrane-bound protein thiols may cause $Ca^{2+}$ efflux and cycling.

The underlying question remains: how does the absence of extracellular $Ca^{2+}$ generate this oxidative stress? It has been demonstrated that superoxide ($O_2^{\cdot-}$) and $H_2O_2$ production by the mitochondria is sensitive to the mitochondrial $Ca^{2+}$ content (100). This may account for an initial oxidant stress capable of inducing $Ca^{2+}$ efflux. Masini et al (99) have verified that mitochondria are sensitive to peroxidative damage by demonstrating that treatment of isolated mitochondria with iron chelates can induce $Ca^{2+}$ efflux and loss of $\Delta\varphi$.

Perhaps the most striking finding from the calcium omission model is the apparent amplification by $Ca^{2+}$ cycling of the initial, perhaps minor, oxidant stress as evidenced by the ability of RR to totally prevent MDA production, GSH oxidation, and vitamin E loss (79, 80). This may be related to the recent observation of Yoshihara & Thurman (101) that RR blocked the stimulation of oxygen uptake by hepatocytes under high oxygen tension, prompting them to suggest that oxygen uptake in hepatocytes may be regulated by $Ca^{2+}$-dependent processes.

The finding that altered cell $Ca^{2+}$ content affects viability of cells differently, depending on the constituent makeup of the extracellular environment, was exploited to examine the relationship of GSH and the glutathione redox cycle during the course of events leading to chemical-induced cell death. In the isolated hepatocyte model system there are several means by which the GSH level and associated enzyme systems can be modulated (78).

Plasma membrane alteration is indicated by the formation of morphological surface protrusions known as blebs, as visualized under scanning electron microscopy (78), and by the diffusion of MDA to the incubation medium as a product of peroxidation of membrane fatty acids (75). Since the extent of injury described by these parameters is reversible (74, 102), it thereby constitutes early stages in the pathway to cell death.

The mechanism whereby lowered cell $Ca^{2+}$ leads to these biochemical and morphological changes is apparently due to the redistribution of intracellular $Ca^{2+}$ stores. Omission of extracellular calcium eradicates the usually high $Ca^{+}$ concentration gradient across the plasma membrane, leading to an initial loss of cytosolic $Ca^{2+}$ as it is continually displaced from the cell (74). Lowering of cytosolic $Ca^{2+}$ is proposed to trigger the release of $Ca^{2+}$ stored in internal membrane sites into the cytosol, thereby raising cytosolic $Ca^{2+}$ above physiological levels, in spite of lowered total cell calcium. Also, a rise in cytosolic $Ca^{2+}$ has been proposed to stimulate calcium-dependent membrane-bound phospholipases (103–105) and to activate certain calcium-dependent nonlysosomal proteases (106). The activation of these enzymes would be expected to alter lipids and proteins of the mitochondrial and plasma

membranes, and, in particular, the cytoskeletal structure, resulting in the loss of mitochondrial function and leaky plasma membranes. During massive oxidative stress, this would lead to cell death.

In a series of reports, Orrenius et al (30, 107) demonstrated that during menadione-induced $H_2O_2$ production in isolated hepatocytes the depletion of intracellular GSH and the modification of key protein thiols result in the perturbation of the normal flux and storage of intracellular $Ca^{2+}$ at internal organelle sites, particularly the mitochondrial and endoplasmic reticular membranes. This results in the release of free $Ca^{2+}$ into the cell cytosol. The thiol-containing plasma membrane $Ca^{2+}$ pumps are also damaged, which may contribute to a decreased ability of the cell to extrude the increased $Ca^{2+}$ load. The net result of this disruption in calcium homeostasis is increased cytosolic $Ca^{2+}$. This reasoning has been extended to earlier studies on the role of extracellular $Ca^{2+}$ in cell death (108–110) whereby an intracellular disturbance in calcium homeostasis, rather than an influx of extracellular $Ca^{2+}$, is considered to have been responsible for the events leading to cell death (30), as described above. Accumulating evidence thus implicates the disturbance of intracellular calcium homeostasis as a major event that triggers membrane-degenerative process within chemically intoxicated cells and has been proposed to be a common mechanism in the pathway to chemically induced cell death (86, 106, 111). However, substantial evidence has been presented equally to claim that increased cytosolic $Ca^{2+}$ concentration plays no major role in cytotoxicity during exposure of hepatocytes to $H_2O_2$ or $CCl_4$ (112, 113).

## ROLE OF MITOCHONDRIA

In the calcium omission model, the internal site of $Ca^{2+}$ storage that is immediately affected by altered cytosolic $Ca^{2+}$ content remains uncertain. Mitochondria are capable of taking up large amounts of $Ca^{2+}$ (114), driven by the proton translocation-generated membrane potential, which is sensitive to RR (93). This has led to the general belief that the mitochondrion can be a major regulator of cytosolic $Ca^{2+}$ concentration. Under pathological conditions, it appears that mitochondrial membrane storage sites are responsible solely for the regulation of intramitochondrial calcium homeostasis (115) since they can apparently maintain extramitochondrial $Ca^{2+}$ concentration only at levels 5–10 times higher than normal cytosolic levels (96).

Although mitochondrial regulation of cytosolic $Ca^{2+}$ concentration appears somewhat untenable, recent observations that RR prevents the oxidative stress and associated cell injury in the calcium omission model (81) does suggest a role for mitochondrial $Ca^{2+}$ cycling in the generation of oxidative cell injury associated with calcium depletion. Mitochondrial GSH is a compart-

mentalized pool of GSH, which is sequestered from cytosolic GSH, and is critical in protection against cytotoxicity (35, 46). After the initial $Ca^{2+}$ redistribution within the calcium-depleted hepatocyte, mitochondrial GSH diminishes in parallel with cytosolic GSH (91, 102). Alterations in the mitochondrial GSH redox system correlate with changes in mitochondrial $Ca^{2+}$ concentration (28), probably as a result of secondary alterations of mitochondrial membrane sulfhydryl groups that are involved in $Ca^{2+}$ retention (116). Both $CCl_4$ and formaldehyde toxicities have been shown to be strongly related to mitochondrial functional changes secondary to changes in mitochondrial thiols and $Ca^{2+}$ concentration (117, 118).

Exposure of renal mitochondria to oxygen free radicals during disruption of calcium homeostasis reduces mitochondrial ATPase activity and other functions that are related to mitochondrial membrane damage (119). Nephrotoxicity of a variety of alkyl and alkenyl halides is mediated by S-conjugates that form $\beta$-lyase-dependent reactive metabolites (for a review see 120). Interaction of these reactive metabolites with the mitochondrial inner membrane, loss of membrane potential, and disturbance of calcium ion homeostasis has been concluded from studies of S-pentachlorobutadienyl-L-cysteine toxicity with isolated rat renal cortical mitochondria (121). Together (119–121), these studies indicate a major, if not critical, role for the mitochondrion in oxidation-associated cell injury that in some instances also involves covalent binding. Covalent binding damage may have similar consequences in acetaminophen toxicity. Acetaminophen administered intraperitoneally to fasted, phenobarbital-induced mice produced hepatotoxicity that included alterations in mitochondrial calcium levels and a decreased ability of isolated mitochondria to sequester calcium. These effects were not produced by 3'-hydroxyacetanilide and covalent binding to mitochondria was much lower with this agent than with acetaminophen (122).

An important aspect of altered mitochondrial functions is the nature of the change in $\Delta\varphi$. Thomas & Reed (79, 80) have observed that RR and $La^{3+}$, which block $Ca^{2+}$ translocation through the mitochondrial uniport, totally prevented MDA formation, GSH and protein thiol oxidation, and $\alpha$-tocopherol loss induced by $Ca^{2+}$ omission. Accordingly, these agents also prevented leakage of intracellular $K^+$. Similar protective effects were provided by the $Ca^{2+}$ chelator EGTA. In these studies, the absence of extracellular $Ca^{2+}$ resulted in a marked decline of the $\Delta\varphi$ which could be prevented by RR, EGTA, vitamin E, and the iron chelator, desferrioxamine. In contrast, oxidative stress induced by treatment with the redox active agent paraquat and BCNU had little effect on $\Delta\varphi$, and MDA formation and $K^+$ leakage were not affected by RR or $La^{3+}$. Oxidative stress induced by $Ca^{2+}$ omission or paraquat and BCNU also led to a marked loss of cellular ATP which was prevented by RR only in the $Ca^{2+}$ omission model. The ability of

vitamin E and desferrioxamine to inhibit the loss of $\Delta\varphi$ indicates that oxidative damage is involved in producing mitochondrial dysfunction. Furthermore, the potent inhibitory effects of RR and $La^{3+}$ suggest that $Ca^{2+}$ movement through the uniport, perhaps indicative of mitochondrial $Ca^{2+}$ cycling, plays a major role in generating this oxidative stress and promoting cell injury (79, 80). Also, because the studies on the oxidative stress induced by paraquat have demonstrated its lack of effect on $\Delta\varphi$, the type of oxidative stress associated with calcium depletion is believed to be fundamentally distinct from that incurred with redox active chemicals (79, 80).



## CHEMICAL-INDUCED CELL INJURY AND MAINTENANCE OF PROTEIN THIOLS

Various investigations have indicated that protein thiols, more so than nonprotein thiols, are critical for the maintenance of cell viability during toxic chemical insult (84, 85, 121). Oxidative stress, which can enhance S-thiolation, causes formation of 1 nmol protein-SSG/1 nmol GSSG during *t*-BuOOH oxidation of mitochondria. This is consistent with the equilibrium constant for the reaction between GSSG and protein thiols, which is close to 1 (123). Iodoacetamide, a protein inhibitor of thioltransferase, did not inhibit the formation of protein glutathione mixed disulfide (protein-SSG) in isolated mitochondria, suggesting that the disulfide formation occurred nonenzymatically or via a different enzyme that was not inhibited by iodoacetamide (45). Although the formation of protein-SSG in the rat lung is a mechanism for maintaining NADPH levels during oxidative stress (124), oxidation of protein thiols has been correlated with increased toxicity of several agents (76, 85, 121), possibly by affecting membrane permeability.

At extremely low concentrations of intracellular GSH, cell viability correlated with the maintenance of protein thiol levels (76). Thus, intracellular $Ca^{2+}$ homeostasis and the mitochondrial thiol redox system for both protein and nonprotein thiols may be closely linked in maintaining cell viability.

## POSSIBLE ROLE OF $\alpha$-TOCOPHEROL IN THE MAINTENANCE OF PROTEIN THIOLS

Calcium omission places stress on the protective systems of the hepatocytes by alteration of intracellular calcium homeostasis and by enhancement of oxidative stress, and thus it affects the contents of cellular thiols and level of $\alpha$-tocopherol. Because toxic chemicals enhance these effects, it is possible to examine the relationships among inhibitors and enhancers of oxidative stress. The stressed hepatocytes become predisposed to the toxic effects of numerous chemicals, including those of such chemical diversity as $CCl_4$, bromo-

benzene, adriamycin (ADR), ethyl methanesulfonate (EMS), and the cationic ionophore A23187 (109, 110). Despite the differences in mechanisms of initiation of toxicity, most of these compounds induce cell membrane damage to isolated hepatocytes (stimulation of lipid peroxidation and production of plasma membrane blebs). Accordingly, protection against the toxicity of EMS, A23187, and ADR/BCNU is proportional to the $\alpha$-tocopherol content of the cells (109).

In addition to $\alpha$-tocopherol, a balanced intracellular thiol redox system is also important for cell viability. A component of this redox system is the status of thiol groups essential for the activities of many enzymes, including the membrane-bound $Ca^{2+}$ translocases (125, 126). It has been proposed that GSH maintains cell viability via the maintenance of membrane protein thiol groups, including those of the critical $Ca^{2+}$ translocases, through thiol-disulfide exchange reactions (30). The relationship between thiol status and $\alpha$-tocopherol during cytotoxicity induced in the calcium omission model has been examined with two chemical protocols that deplete intracellular GSH by different mechanisms. One involved the indirect depletion of GSH by ADR-mediated generation of reactive oxygen species. ADR, an anthracycline quinone compound, is known to undergo redox cycling between the quinone and the semiquinone radical to produce $H_2O_2$ and $O_2^{\cdot-}$ (127, 128). $H_2O_2$ is reduced to $O_2$ and $H_2O$ by GSH peroxidase, with the oxidation of GSH cofactor to GSSG. Since ADR-mediated depletion of GSH is only observed during the inhibition of GSH regeneration, BCNU was added along with the quinone to inhibit GSH reductase (20). The second mechanism of GSH depletion utilized ethacrynic acid (EA) to directly conjugate intracellular GSH (129).

EA (100 $\mu$M) or ADR (350 $\mu$M)/BCNU (100 $\mu$M) lowered intracellular GSH to nondetectable levels in hepatocytes at 1 hr, and to less than 5% of initial levels at 3 hr, respectively (77). GSH decline was accompanied by decreased cellular $\alpha$-tocopherol content and followed by losses in protein thiol levels as well as cell viability. Although depletion of GSH was independent of cell $Ca^{2+}$ concentration, the losses in all three parameters were maximal in calcium-depleted cells. Supplementation with $\alpha$-tocopherol succinate to both calcium-depleted and calcium-adequate cells elevated cellular $\alpha$-tocopherol levels, but, in contrast to that observed in cells exposed to the simple $Ca^{2+}$ omission-induced stress described above, intracellular GSH concentrations were unaffected by $\alpha$-tocopherol succinate treatment and remained below detectable levels. A similar lack of antioxidant ability to maintain intracellular GSH during its chemical depletion and stimulation of lipid peroxidation was not unexpected and had been observed by others (130).

In spite of this severe depletion of GSH, protein thiol levels continued to reflect the $\alpha$-tocopherol content of the cells, such that at the highest $\alpha$-

tocopherol content (approximately 1.0 nmol/$10^6$ cells in calcium-depleted cells), protein thiol remained above 75% of initial levels. At these levels of $\alpha$-tocopherol and protein thiol, the toxic effects of GSH-depleting agents were completely prevented (76). By loading both calcium-depleted and calcium-adequate cells with equal intracellular concentrations of $\alpha$-tocopherol (0.8–1.0 nmol/$10^6$ cells) during the 5-hr incubation period, both protein thiol content and cell viability were maintained at initial levels regardless of the depleted intracellular GSH stores or the status of cell calcium (77). Thus, during the total depletion of intracellular GSH, the levels of protein thiol in isolated hepatocytes, in parallel with cell viability, correlate with cellular $\alpha$-tocopherol content. Although depletion of intracellular GSH levels below 20% of physiological levels in general has been considered to be detrimental to the cell (38, 41, 72, 131), these recent findings place further limits on the significance of the GSH threshold when the $\alpha$-tocopherol level is elevated (132).

The observation that cell viability directly correlated with protein thiol levels strongly supports the hypothesis of Orrenius and coworkers that loss of protein thiol groups is one of the critical factors leading to cell death (106). However, in their experimental model, chemical modulation of protein thiol alters the mechanisms involved in the maintenance of calcium homeostasis, thereby increasing cytosolic $Ca^{2+}$ concentration—believed by them to be the ultimate step leading to damage of the cytoskeletal structure and cell membranes (86). This compares to the present model whereby, regardless of altered calcium homeostasis, maintenance of protein thiol groups prevents the loss of cell integrity. Human platelets exposed to the redox-active quinone, menadione, undergo cytoskeletal alterations that are mediated by metabolically induced oxidation and depletion of thiols as well as ATP depletion (133). Both oxidative and calcium ion-dependent mechanisms are implicated in the increase in amount of cytoskeleton-associated protein and loss of protein thiols. High ATP levels correlated with prevention of menadione-induced increase in cytosolic calcium ion (133). Addition of dithiothreitol (DTT) solubilized a considerable amount of the polypeptides associated with the cytoskeletal fraction isolated from hepatocytes exposed to quinone, including menadione (134).

Interestingly, loss of cellular protein thiols can be prevented by a wide variety of antioxidants and metal in chelators, all of which control free radical-mediated oxidative events. It is unclear whether $\alpha$-tocopherol-mediated maintenance of protein thiols prevents the release of compartmentalized $Ca^{2+}$ into the cell cytosol during lowered total cell calcium concentrations and thereby prevents associated membrane degradative processes, or whether damage is prevented in spite of elevated cytosolic $Ca^{2+}$ concentration.

The hypothesis that α-tocopherol prevents oxidative stress and associated injury by maintenance of protein thiol has been only alluded to by other investigators using erythrocyte models of oxidative stress (135–137). The ability of α-tocopherol to prevent oxidative damage to an integral membrane protein has received very little attention, even though it has been noted that vitamin E deficiency alters the activities of numerous membrane-bound enzymes (138). For example, in rabbit skeletal muscle, α-tocopherol has been shown to stabilize $Ca^{2+}$-ATPase activity of sarcoplasmic reticulum and prevent the loss of transport of $Ca^{2+}$ ions during damage by exposure to thermal-irradiated unsaturated fatty acids (139). The loss of activity of this enzyme by oxidizing agents has been advocated to result from the direct oxidation of SH groups, rather than being secondary to lipid peroxidation (140). It would appear then that α-tocopherol maintains this $Ca^{2+}$ ATPase activity via direct maintenance of thiols and is not secondary to its inhibition of lipid peroxidation. Whichever mechanism is operative, the close similarity between this enzyme and that of the endoplasmic reticulum suggests a commonality in α-tocopherol action.

## S-THIOLATION OF PROTEIN IN CELLS

Increased oxidation of GSH to GSSG in vivo can promote protein S-thiolation with the protein mixed disulfide being formed presumably by thiol/disulfide exchange. The rate of thiol/disulfide exchange of protein thiols is related to the reactivity of each specific protein thiol with the oxidized thiol (141). Reactivity of each protein thiol is a property of the overall chemical nature of the thiol reaction site. In addition to protein disulfides, low molecular weight disulfides that may participate in vivo are GSSG, cystine, and cystamine. Cellular S-thiolation has been shown to occur with four enzymes, glycogen phosphorylase, (141, 142) creatine kinase (142), a proteinase inhibitor (143), and glyceraldehyde 3-phosphate dehydrogenase (GPD) (144).

Dethiolation of S-thiolated protein depends on the reaction between S-thiolated protein and one or more reduced thiols. GSH could have a key role in the maintenance of protein thiols in their dethiolated forms. If so, then maintenance of GSH/GSSG redox status is important to the degree of S-thiolation of those protein thiols that are sufficiently reactive to undergo thiol/disulfide exchange processes.

The reduction of protein mixed disulfide (dethiolation) has been examined with thioredoxin as the thiol reductant. Electrofocusing methodology was used to determine the rate of dethiolation of glycogen phosphorylase b and creatine kinase (145). Because thioredoxin displays a broad specificity for reduction of disulfide bonds in low-molecular-weight disulfides and protein

disulfides and is present in almost all mammalian cells, it appears to have an important role in dethiolation of specific proteins (145).

The enzymatic activity of the thiol-dependent enzyme, glyceraldehyde 3-phosphate dehydrogenase in vertebrate cells can be modulated tenfold by a change in the intracellular thiol/disulfide redox status (146). Human lung carcinoma cells (A549), which contain high levels of GPD, lost 90% of GPD activity when incubated with appropriate levels of hydroperoxides or thiol reagents. Loss of protein thiol and loss of GPD enzymatic activity occurs in a dose-dependent manner.

Following oxidant treatment, DTT partially reversed loss of thiol and enzymatic activity of cellular GPD. Cellular recovery of GPD enzymatic activity after oxidant treatment occurred without addition of low-molecular-weight thiols, however, at a slower rate than if thiols were added to the incubation medium. Thioredoxin and GSH-dependent processes may contribute to the reduction of the essential thiol group that is lost due to oxidant exposure (146).

## MICROSOMAL AND NUCLEAR GSH-DEPENDENT PEROXIDASES

GSH, either alone (147) or in conjunction with added protein (148, 149), can protect microsomes against lipid peroxidation. Addition of GSH produced a 40% reduction in NADPH-induced peroxidation without addition of rat liver microsomes or other proteins (150). A membrane-bound glutathione S-transferase possessing glutathione peroxidase activity has been purified from rat liver microsomes (151). After solubilization, the enzyme, which has a monomer molecular weight of 14,000, can reduce cumene hydroperoxide. This enzyme appears unique with respect to soluble GSH S-transferases in that it is activated by NEM (152). A microsomal glutathione S-transferase has been purified from human liver and found to have characteristics similar to those of the rat microsomal S-transferase as described by Morgenstern & De Pierre, (151) and McLellan et al (153).

Glutathione S-transferase activity present in rat liver microsomes may be regulated by reversible thiol/disulfide exchange. Activation of microsomal glutathione S-transferase by diamide or cystamine can be reversed by the addition of DTT whereas GSSG increased the activity of only membrane-bound enzyme (154).

Mouse liver microsomal glutathione S-transferase has been purified in the NEM-activated as well as in an inactivated form. Inhibitor studies indicate a cooperative effect involving conversion of activated enzyme into the inactivated form (155). Lipid peroxidation inhibits microsomal glutathione S-

transferase from rat, and such inhibition appears modulated by dietary antioxidants (156).

The existence of a membrane-associated peroxidase has been demonstrated by the purification of a selenium-containing glutathione peroxidase from pig heart (148). The enzyme is distinct from the classical glutathione peroxidase by virtue of differences in substrate specificity requirements even though the similar amino acid composition was noted (148).

Administration of allyl alcohol to rats leads to an activation of microsomal glutathione S-transferase; however, a reduction of the GSH-dependent protection against lipid peroxidation also occurs. Haenen et al have proposed that the activation of glutathione S-transferase in vivo by allyl alcohol proceeds via alkylation of thiol group of the glutathione S-transferase by acrolein (157). Further, these workers speculate that acrolein decreases the GSH-dependent protection against lipid peroxidation by alkylation of the thiol(s) group on a proposed vitamin E free radical reductase. In this regard, Gibson et al (149) have reported that a cytosolic, GSH-dependent protein can protect microsomal membranes against peroxidation. GSH did not appear to be utilized in glutathione peroxidase activity but rather in the inhibition of the initiation of peroxidation—possibly by serving in a reduction system for $\alpha$-tocopherol (149). A recent report provides indirect evidence for a rat liver, free radical reductase in mitochondria and microsomal membranes that prevents vitamin E (chromanoxyl) radical accumulation (158). GSH increased the efficacy of NADPH in preventing the accumulation of the chromanoxyl radical, but was without effect in the absence of NADPH. Since NADH, NADPH and ascorbate prevent the accumulation, these workers speculate that microsomes and mitochondria have both enzymatic and nonenzymatic mechanisms for reducing the chromanoxyl radical of vitamin E. These findings support the earlier speculation that GSH delays microsomal lipid peroxidation via the reduction of the chromanoxyl radical of vitamin E which purportedly is catalyzed by a free radical reductase (159), and they explain why lipoic acid is limited to a nonenzymatic mechanism for protection against microsomal lipid peroxidation (160).

Little is known about the susceptibility of the cell nucleus to lipid peroxidation and possible damage to nuclear nucleic acids. The nuclear membrane regulates the transport of mRNA into the cytoplasm and aids in the process of nuclear division. Since DNA is associated with certain regions of the nuclear membrane (161), nuclear membrane peroxidation may disrupt many of these critical functions. The proximity of the nuclear membrane to DNA could contribute to the interactions of DNA with reactive intermediates generated in lipid peroxidation and known to be mutagenic and carcinogenic (162).

A protection system that prevents or limits oxidative events in the nucleus has been reported. The threshold or minimum levels of vitamin E necessary to

control lipid peroxidation and the nature of the possible "sparing" effects that GSH and vitamin E may have during lipid peroxidation protection are related via GSH-dependent peroxidase activity (163–165). Endogenous $\alpha$-tocopherol levels in isolated rat liver nuclei were measured and found to be 0.045 mole % (mole $\alpha$-tocopherol per mol phospholipid $\times$ 100). This value corresponds to 970 polyunsaturated fatty acid moieties to one molecule of $\alpha$-tocopherol in the nuclear membrane. Exogenous $\alpha$-tocopherol when incorporated in isolated rat liver nuclei above a threshold level of 0.085 mole % effectively inhibited NADPH-induced lipid peroxidation. Addition of GSH lowered the threshold levels of $\alpha$-tocopherol needed to inhibit lipid peroxidation to about 0.04 mole %. GSH protected against lipid peroxidation by inducing a lag period prior to onset of peroxidation. This GSH-induced lag period was abolished by treatment of nuclei with trypsin, thiol reagents, disulfides, or heating nuclei at 60 °C for 15 min, which destroys the peroxidase activity.

In the presence of GSH, isolated nuclei catalyzed the conversion of cumene hydroperoxide to cumyl alcohol but failed to metabolize $H_2O_2$. GSH S-transferases display such a substrate specificity pattern (166, 167). The GSH-dependent peroxidase activity and inhibition of lipid peroxidation were abolished by exposing isolated nuclei to the GSH S-transferase inhibitor S-octylglutathione (165). S-Octylglutathione abolished, as well, the conjugation of chloro-dinitrobenzene with GSH and GSH-dependent reduction of cumene hydroperoxide. About 70% of the glutathione S-transferase activity associated with isolated nuclei was solubilized with 0.3% Triton X-100. This solubilized glutathione S-transferase was partially purified by utilizing a S-hexylglutathione affinity column. The partially purified nuclear glutathione S-transferase exhibited glutathione peroxidase activity towards lipid hydroperoxides in solution. Glutathione S-transferase activity associated with the nucleus, which may contribute to the GSH-dependent protection of isolated nuclei against lipid peroxidation, is distinct from the microsomal glutathione S-transferase activity (168).

## CONCLUSIONS

The relationship between GSH and cell viability has received much scientific inquiry. Unfortunately, many investigations that report glutathione depletion as related to some cellular or biochemical endpoint have failed to provide a clear explanation of the role of glutathione in the maintenance of cellular integrity. Rarely are chemical intoxications understood in terms of the events that occur when cell injury becomes so intense that irreversible changes cause cell death. Events leading to cell death will involve the loss of energy production and $\Delta\varphi$, not merely ATP depletion, loss of ionic homeostasis (such as calcium ion regulation), and a complex series of events that involve

the redox status of both pyridine nucleotides and thiols. Oxidative stress, both endogenous and exogenous that includes lipid peroxidation and other peroxidative and hydrolytic processes, may occur depending on the type of chemical agent. These latter events are related in some intoxications to alterations in oxygen metabolism and the lack of calcium ion control needed to prevent modulation of calcium-dependent enzymes.

An emerging area of research inquiry is the pathological implications of loss of mitochondrial functions and the role of protein thiols in the maintenance of cellular integrity. GSH has important cellular relationships to protein thiols even though little can be said about them due to limited information. It is possible to speculate, however, that eventually an even greater amount of research will be focussed on protein thiols than is now focussed upon GSH. These studies will require us to learn much more about the specificity of protein thiol reactivity and participation in both alkylating and oxidant events at the cellular and subcellular levels. The paucity of knowledge about the molecular events that influence the status of protein thiols within the cell and, particularly within the mitochondria and nucleus, seriously limits our ability to understand the roles of both protein and nonprotein thiols. The kinetics of maintenance of thiol homeostasis, which is energy-driven, along with calcium ion regulation can have a significant impact on the total energy-generation capacity of intoxicated cells and in turn on the survival of such cells. Future research will need to address the question of the dynamics of homeostasis of thiols and the rate of energy consumption for cellular processes related to such homeostasis during pathological conditions.



ACKNOWLEDGMENTS

This review was supported in part by NIEHS grant ES01978 and grant CH-109 from the American Cancer Society.

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